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Insight from Molecular, Pathological, and Immunohistochemical Studies on Cellular and Humoral Mechanisms Responsible for Vaccine-Induced Protection of Rainbow Trout against *Yersinia ruckeri*

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The immunological mechanisms associated with protection of vaccinated rainbow trout, *Oncorhynchus mykiss*, against enteric redmouth disease (ERM), caused by *Yersinia ruckeri*, were previously elucidated by the use of gene expression methodology and immunochemical methods. That approach pointed indirectly to both humoral and cellular elements being involved in protection. The present study correlates the level of protection in rainbow trout to cellular reactions in spleen and head kidney and visualizes the processes by applying histopathological, immunohistochemical, and *in situ* hybridization techniques. It was shown that these cellular reactions, which were more prominent in spleen than in head kidney, were associated with the expression of immune-related genes, suggesting a Th2-like response. *Y. ruckeri*, as shown by *in situ* hybridization (ISH), was eliminated within a few days in vaccinated fish, whereas nonprotected fish still harbored bacteria for a week after infection. Vaccinated fish reestablished normal organ structure within a few days, whereas nonprotected fish showed abnormalities up to 1 month postinfection. Protection in the early phase of infection was mainly associated with the expression of genes encoding innate factors (complement factors, lysozyme, and acute phase proteins), but in the later phase of infection, increased expression of adaptive immune genes dominated. The histological approach used has shown that the cellular changes correlated with protection of vaccinated fish. They comprised transformation of resident cells into macrophage-like cells and increased occurrence of CD8α and IgM cells, suggesting these cells as main players in protection. Future studies should investigate the causality between these factors and protection.

Vaccine development is an integral and ultimate aim of any vaccine research and requires an array of immunological and pathological detection assays for the evaluation of immune protection. The expression of relevant immune gene markers should be combined with a functional approach correlating protection with the presence of effector molecules and morphopathological alterations in order to demonstrate vaccine-mediated interference in disease processes (1). Following the immunization of rainbow trout with a vaccine against enteric redmouth disease (ERM) and subsequent challenge with *Yersinia ruckeri*, it has been shown that genes encoding various cytokines, chemokines, cell marker- and immune-related proteins (acute-phase reactants, immunoglobulin and complement, and lysozyme) become activated (2, 3) in central immune organs. Humoral responses reflected by increased plasma antibody and lysozyme levels have also been documented (1, 4–6). However, limited information is available on the dynamic cellular processes associated with protection, due to a lack of anatomical and histopathological investigations of central immune organs following vaccination and infection. Classical histopathological studies in fish exposed to bacteria, including *Y. ruckeri*, have placed their focus on pathogenesis in various tissues and organs without addressing the differential and sequential immune cell involvement and dynamics in central lymphoid organs (7, 8). Considering the scarcity of flow cytometric assays in vaccinated fish (9–11), due to a lack of immunological reagents (monoclonal antibodies reacting with native cell markers) (12–16), we were encouraged to undertake descriptions of the morphopathological alterations/modifications of immune organs following challenge. This approach would suggest specific cell types as being active during the development of immunity toward ERM. Immune cells could be identified by immunohistochemistry (IHC) using our newly developed monoclonal antibodies (17), and their possible qualitative and quantitative changes following challenge could be correlated to the expression of immune genes and the presence of effector molecules in serum and tissue. In brief, we visualize herein the sequential development of cellular alterations in two central immune organs (spleen and head kidney) of fish vaccinated against ERM and unvaccinated fish. The differences noted are correlated to protection, the expression of immune-related genes, and the presence of effector molecules, allowing a sequential description of immune processes and suggesting future investigations to clarify the causality, if any, between protection and the involvement of the observed cells.

MATERIALS AND METHODS

Experimental animals. Rainbow trout eggs from the certified virus-free Fousing trout farm, Jutland, Denmark, were transferred to a pathogen-free hatchery, Aqua Baltic, in Nexø, Denmark. The eggs were disinfected, incubated, and hatched, and fish fry were initially reared at 7°C until transfer to 1-m^2 tanks (volume, 700 liter each) where the temperature was gradually increased from 7 to 13°C over 2 months. After attaining the juvenile stage (90 days posthatching [1,170 degree days]), the rainbow

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TABLE 1 Experimental setups

Time (mo) postvaccination (degree days)	Group	No. of fish[a]	Inoculum (100 μl i.p.)	Challenge (no. of CFU/fish)	RPS[b]	Sampling time(s) postchallenge (days)[c]	Assays performed[d] (no. of fish/assay)
4 (2,730)	Naive	20	None	None	None	0	SI (3), LA (5), Ab ELISA (5), RT-PCR (5)
	Sham challenged	20	Sterile PBS	None	None	3, 7, 21, 30	HP (2 or 3), IHC (2 or 3), ISH (2 or 3)
	Nonimmunized infected	80	PBS + *Y. ruckeri*	8×10^6	73.7[e]	3, 7, 21, 30	
	AquaVac ERM vaccinated	80	PBS + *Y. ruckeri*	8×10^6	13.5	0, 3, 7, 21, 30	
	AquaVac RELERA vaccinated	80	PBS + *Y. ruckeri*	8×10^6	42.3	0, 3, 7, 21, 30	
8 (4,290)	Naïve	12	None	None	ND	0	SI (3), LA (5), Ab ELISA (5), HP (2 or 3),
	Sham challenged	20	PBS	None	ND	3, 7, 14	IHC (2 or 3), ISH (2 or 3)
	Nonimmunized infected	35	PBS + *Y. ruckeri*	6.3×10^7	ND	3, 7, 14	
	AquaVac ERM vaccinated	35	PBS + *Y. ruckeri*	6.3×10^7	ND	0, 3, 7, 14	
	AquaVac RELERA vaccinated	35	PBS + *Y. ruckeri*	6.3×10^7	ND	0, 3, 7, 14	

[a] Number of fish per duplicate tank.
[b] RPS, relative percentage of survival; ND, not done. RPS = (1 − [percent mortality in vaccinates]/[percent mortality in the control and nonimmunized group]) × 100.
[c] Day 0 was the day before challenge; 21-day samples were used for histopathology.
[d] SI, spleen index; LA, lysozyme assay; HP, histopathology.
[e] % mortality.

trout (1,800 in total) were randomly segregated to six parent tanks (i.e., three duplicate groups with 300 fish per tank) (18). Degree days are computed as the integral of a function of time that generally varies with temperature. Temperature controls the development rate of many organisms, including fish. All fish were fed equally at 1.0% of biomass per day with standard dry pelleted feed (BioMar A/S Brande, Denmark).

Vaccines and immunization. Fish were immunized as previously described (18). Two commercial vaccines, i.e., AquaVac ERM (a bacterin consisting of formalin-inactivated *Y. ruckeri* biotype 1 at 5×10^9 CFU/ml) and AquaVac RELERA (a bacterin with *Y. ruckeri* biotype 1 at 5×10^9 CFU/ml and EX5 biogroup *Y. ruckeri* biotype 2 at 5×10^9 cells/ml), were used in the study. The randomly categorized juvenile rainbow trout (mean body weight of 5 g) were immunized by immersion into a diluted bacterin (1:10) for 30 s according to the manufacturer's instructions. The control fish were immersed (sham vaccinated) in water for the same duration. The fish were then maintained at the same rearing facility at 12 to 13°C after vaccination until transportation at 4 months postvaccination (m.p.v.) (2,730 degree days) or 8 m.p.v. (4,290 degree days) to our infection facility at the University of Copenhagen, where they were challenged (18).

Bacterial strain and growth conditions. *Y. ruckeri* strain 100415-1/4 (serotype O1, biotype 2) which was originally obtained from a clinical outbreak of ERM in a freshwater trout farm, was utilized for all challenge studies. The bacterium was identified (19), cultured in Luria-Bertani (LB) broth, and incubated on an orbital shaker at 22°C for 48 h until reaching peak exponential phase, at which time the culture was harvested via centrifugation (at 22°C and 4,000 × *g* for 10 min) and serial 10-fold dilutions were prepared with sterile phosphate-buffered saline (PBS) to determine the bacterial cell concentration (i.e., CFU) by the plate count method.

Bacterial challenge and sampling. Animal procedures were performed under the guidelines of and permission was obtained from the Committee for Experimental Animals, Ministry of Justice, Denmark. After they arrived at the university fish-keeping facility, the fish were tested and confirmed free of bacterial (head kidney swab examination) and parasitic infestation (20). At every challenge trial, before infection, head kidney swabs of five randomly selected fish from each duplicate group were tested on blood agar plates in an attempt to analyze and confirm that the fish were free from bacterial infection. Two challenge experiments were carried out (18), and the fish used were grouped as shown in Table 1.

We used intraperitoneal injection in order to ensure uniform exposure of each fish to the pathogen. The challenge dose was increased in older fish in accordance with the conditions for potency testing of fish vaccines suggested previously (21). Fish were euthanized before sampling by exposure to an overdose of tricaine methane sulfonate (MS-222; 300 mg/liter).

Spleen somatic index. The spleen somatic index was calculated as the spleen weight (mg) divided by the whole-body weight (g), as described previously (22).

Serum lysozyme activity. Serum lysozyme activity was estimated by a turbidimetric assay (23) with a few modifications (24) at 25°C. Here, one unit of lysozyme activity was defined as a 1% decrease in absorbance during an incubation time of 4 min, calculated with the formula $[(OD^{1\ min} - OD^{5\ min})/OD^{1\ min}] \times 100$.

ELISA for determination of specific *Y. ruckeri* antibody. The level of specific anti-*Y. ruckeri* serum antibody reactivity was determined by enzyme-linked immunosorbent assay (ELISA) as recently described (25). Dilution series of fish sera ($1, 10^{-1}, \ldots 10^{-4}$) were produced, and volumes of 100 μl of diluted sera (in duplicate) were considered for the assay. In each assay, sera from fish that were hyperimmune to *Y. ruckeri* and dilution buffer were used as the positive and negative control, respectively. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (catalog number STAR13B; AbD Serotec, Germany) was used as the tertiary antibody. Mouse anti-salmonid IgG (catalog number MCA 2182; AbD Serotec), which reacted with trout Ig binding to the antigen *Y. ruckeri* bound to the 96-well microtiter plate, was used as the secondary antibody. Tetramethylbenzidine (TMB) substrate (TMB plus, catalog number BUF042B; AbD Serotec) was used for color development, and the reaction was stopped by adding 1 M HCl. The resulting value for the optical density at 450 nm (OD_{450}) was obtained with a plate reader (Epoch; Biotek Instrument, Inc.). All OD_{450} results presented were from 1:100 dilutions (showing the best resolution), and a cutoff value of 4 was applied when the saturation was exceeded.

RNA isolation and cDNA synthesis. Samples of three tissues, *viz.*, liver, spleen, and head kidney, were homogenized using a FastPrep-24 instrument (MP Biomedicals, Solon, OH), and total RNA was isolated using a GenElute mammalian total RNA kit (RTN-350; Sigma-Aldrich, Denmark). Before reverse transcription, the RNA concentration in all samples was adjusted to 200 ng using RNase-/DNase-free water (Gibco, Invitrogen, Denmark), and the RNA was transcribed to cDNA using random hexamers (TaqMan reverse transcription, catalog number 4322235; Applied Biosystems, Denmark). The reactions were performed in a Bio-Rad T100 thermal cycler using a 20-μl reaction mixture volume under the following conditions: 25°C for 10 min to anneal primers, 37°C for 60 min for elongation, and 95°C for 5 min (inactivation). The resultant cDNA samples were diluted 10 times by adding 180 μl of

RNase-/DNase-free water (Gibco, Invitrogen, Denmark) and stored at −20°C until the next use.

qPCR. Real-time quantitative PCR (qPCR) assays were performed using a Stratagene MX 3005P (Agilent Technologies, United States). Samples totaling 2.5 μl of diluted cDNA (200 ng) were used as the templates in 12.5-μl qPCR reaction mixtures (total volume) with Brilliant II qPCR master mix (6.25 μl), 10 μM forward primer (0.5 μl), 10 μM reverse primer (0.5 μl), 5 μM TaqMan probe (0.5 μl), and RNase-/DNase-free water (Gibco; Invitrogen, Denmark) (2.25 μl). The cycling conditions were 1 cycle of 95°C for 15 min for predenaturation, followed by 45 cycles of 95°C for 15 s and 60°C for 45 s (combined annealing and elongation step). Several controls were included in each qPCR plate setup. Thus, control wells with no template and control wells with no reverse transcriptase (in duplicate) were used (6, 17). The primers and probes used are shown in Table 2.

Histopathology and scoring. Tissue samples collected in 10% neutral buffered formalin (for 24 to 48 h) were subsequently transferred to 70% ethanol until processing. The tissues were dehydrated through graded ethanol baths and xylene and embedded in paraffin wax. Sections 4 to 5 μm thick were cut with the help of a microtome (Leica 2125; Leica, Germany) and stored at 4°C until further use. For histopathological evaluation and to study the distribution of immune cells, spleen and head kidney samples were considered. To detect *Y. ruckeri* antigen in fish after challenge, the following additional organs were also sampled and processed: liver, posterior kidney, heart, gills, and intestine. A semiquantitative scoring system was followed for spleen and head kidney based on the presence of pathomorphological alterations (Tables 3 and 4). The alterations in organ structure were categorized under four broad parameters: (i) damage to the organ, (ii) reactive change with a negative impact, (iii) susceptibility, and (iv) protective cellular response. The scores for these broad parameters were combined to address the overall degree of tissue changes seen in each organ/fish examined.

Staining techniques. Hematoxylin and eosin (H&E) staining was carried out for histopathology interpretation (26).

IHC for recording immune cell distribution and detection of *Y. ruckeri* antigen. Immunohistochemistry was used for visualization of $CD8\alpha^+$ and IgM^+ cells in spleen and head kidney as previously described (17). Similarly, IHC detected *Y. ruckeri*-specific antigen in formalin-fixed and paraffin-embedded multiorgan sections using a mouse monoclonal antiserum as previously described (27). The antibodies used in immunohistochemistry were (i) a monoclonal antibody (MAb) raised against salmon CD8α (dilution, 1:300) (28), (ii) a MAb against salmon IgM (dilution, 1:150) (29), and (iii) a MAb raised against a Chilean isolate of *Y. ruckeri* (dilution, 1:5,000) (27).

Scoring of immune cells. The staining intensities, densities, and distribution of positive $CD8\alpha^+$ and IgM^+ cells were assessed at ×100 magnification on a scale from 0 to 4, where 0 indicates no presence or staining of cells and 4 indicates extensive presence and marked staining of positive cells. IgM staining appeared as two different patterns, i.e., stained positive cells (IgM^+ cells) and a diffuse intercellular form (secreted IgM) (Tables 5 and 6). *Y. ruckeri* antigen distribution was scored at ×100 magnification, according to a scale from 0 to 7, where 0 represents no presence and 7 represents extensive and diffuse presence in a whole-organ section (Table 7).

FISH. The fluorescent *in situ* hybridization (FISH) technique with a Cy3-labeled 16S rRNA-specific probe for *Y. ruckeri* (30) was performed as recently described (27) to detect the localization and density of the pathogen in the organ (spleen and head kidney). For counting of intact bacteria, the microscopic images were captured and saved as image files. A magnification of ×400 was chosen because the on-screen image showed high contrast and resolution at this magnification. The visual field area was measured (0.014 mm^2), allowing bacterial density calculations, and images were taken by moving the stage platform horizontally to both central and peripheral sections of spleen and head kidney. Three regions of each section were counted, and the mean numbers of individual bacteria, as well as both macro- and microcolonies (foci of aggregated bacteria), were calculated.

Statistical analyses. For relative and differential gene expression analysis, the cycle threshold ($2^{-\Delta\Delta CT}$) method was used (31). Gene expression data were only considered significantly up or down regulated when the *P* value was <0.05 and a 3-fold change was obtained. To estimate the relative levels of *Y. ruckeri*, the ΔC_T value was calculated as the difference between the expression levels of trout ELF1α and *Y. ruckeri* 16S. C_T values above 36 were considered negative (no C_T) as previously described, with some modification (32). The $\Delta\Delta C_T$ value was in this case calculated as the difference between the results for the nonimmunized infected group (i.e., *Y. ruckeri*) and the immunized and infected groups. The results are presented as the percentages of the bacterial levels in nonimmunized infected fish. Data such as the spleen index, lysozyme assay, ELISA, and *Y. ruckeri* antigen distribution results were normally distributed and analyzed by one-way analysis of variance (ANOVA) followed by a multiple-comparison test (Tukey's *post hoc* test). For nonparametric data (histopathology score and immune cell distribution), the Kruskal-Wallis one-way ANOVA was performed with the Dunn multiple-comparison test. All data were expressed as means ± standard errors of the means (SEM), and *P* values of <0.05 were considered statistically significant. GraphPad Prism 4.0 was used.

RESULTS

We noted higher survival rates for AquaVac RELERA-vaccinated fish (relative percentage of survival [RPS], 42.3%; mortality, 42.5%) than for AquaVac ERM vaccinates (RPS, 13.5%; mortality, 63.7%) at 4 m.p.v. (18). The cumulative percentage of mortality for the nonimmunized *Y. ruckeri*-infected group was found to be 73.7% (Table 1). Challenge at 6 m.p.v. showed higher levels of survival in AquaVac RELERA-vaccinated fish (RPS, 52%; mortality, 42%); an intermediate level of protection was seen in AquaVac ERM vaccinates (RPS, 29.5%; mortality, 62%). The nonimmunized *Y. ruckeri*-infected group showed cumulative mortality of 88% (18).

Spleen index. At 4 m.p.v., spleen index increases were observed from day 7 postinfection (p.i.) in nonimmunized *Y. ruckeri*-infected fish, while they occurred already from day 3 p.i. in vaccinated fish (exposed to bacteria), with a further marginal increase until day 30 p.i. Significant differences were found between groups at 4 m.p.v. ($P < 0.05$) but not at 8 m.p.v. (data not shown).

Serum lysozyme activity. Prior to infection, no significant differences in serum lysozyme activity were found between any groups at both 4 m.p.v. and 8 m.p.v. (Fig. 1A and B). Following challenge at 4 m.p.v., increases in lysozyme activity were observed in all infected groups at days 3 and 7 p.i. (compared to the activity in sham-challenged control fish), but vaccinated fish showed significantly superior production at day 7 p.i. At day 30 p.i., decreases in lysozyme activity were observed in all infected groups. Variations were seen at 8 m.p.v. Following marked rises at day 3 p.i. in nonimmunized *Y. ruckeri*-infected fish and AquaVac RELERA-vaccinated fish compared to the levels in sham-challenged and AquaVac ERM-vaccinated fish, the activity levels declined in all infected groups at day 7 p.i. and day 14 p.i. No significant difference among groups was observed at day 14 p.i.

Serum antibody responses determined by ELISA. At 4 m.p.v., both nonimmunized *Y. ruckeri*-exposed fish and AquaVac RELERA-vaccinated fish exhibited significant, 3-fold rises in serum antibody levels (at day 30 p.i.) compared to the levels in sham-challenged fish (Fig. 2A and B). AquaVac ERM-vaccinated fish showed intermediate antibody reactions. However, at 8 m.p.v., merely mild differences were noted among vaccinated

TABLE 2 Primers and probes used for real-time quantitative PCR analysis of gene expression

Gene	Direction	Primer	Probe (5′–3′)	Product size (bp)	GenBank accession no.
C3	Forward Reverse	ATTGGCCTGTCCAAAACACA AGCTTCAGATCAAGGAAGAAGTTC	AGCTTCAGATCAAGGAAGAAGTTC	85	AF271080
C5	Forward Reverse	TGGCAAGGACTTTTTCTGCT AGCACAGGTATCCAGGGTTG	CTGGCAGGGATTGCATCAAATC	64	AF349001
SAA	Forward Reverse	GGGAGATGATTCAGGGTTCCA TTACGTCCCCAGTGGTTAGC	TCGAGGACACGAGGACTCAGCA	79	AM422446
Precerebellin	Forward Reverse	TGGTGTTGCTTTGCTGTTGT GCCACTTTTGGTTTGCTCTC	ATGGTTGAGACTCAGACGGAGAGTG	116	AF192969
Cathelicidin 2	Forward Reverse	AAAGATTCCAAGGGGGGT CAAAGGGTGTGTTGTGCTGT	GCTCTCGTCCTGGGTTTGGCTCC	135	AY360356
Lysozyme	Forward Reverse	GAAACAGCCTGCCCAACT GTCCAACACCACACGCTT	ATACCCAGGCCACCAACCGCAACAC	188	X59491
IL-1β	Forward Reverse	ACATTGCCAACCTCATCATCG TTGAGCAGGTCCTTGTCCTTG	CATGGAGAGGTTAAAGGGTGGC	91	AJ223954
IL-6	Forward Reverse	ACTCCCCTCTGTCACACACC GGCAGACAGGTCCTCCACTA	CCACTGTGCTGATAGGGCTGG	91	DQ866150
TNF-α	Forward Reverse	GGGGACAAACTGTGGACTGA GAAGTTCTTGCCCTGCTCTG	GACCAATCGACTGACCGACGTGGA	75	AJ277604, AJ401377
IL-10	Forward Reverse	CGACTTTAAATCTCCCATCGAC GCATTGGACGATCTCTTTCTTC	CATCGGAAACATCTTCCACGAGCT	70	AB118099
CD4	Forward Reverse	CATTAGCCTGGGTGGTCAAT CCCTTTCTTTGACAGGGAGA	CAGAAGAGAGAGCTGGATGTCTCCG	89	AY973028
CD8	Forward Reverse	ACACCAATGACCACAACCATAGAG GGGTCCACCTTTCCCACTTT	ACCAGCTCTACAACTGCCAAGTCGTGC	74	AF178054
GATA-3	Forward Reverse	TCCTGGAGAGAGGGATGAAA AGCCCGAGACCTATAGCACA	GGCCTTCACTTTCGCCTGCT	161	FM863826
T-bet	Forward Reverse	TTCTGCCATTTTGTGTCAGG TTCTCCATCCTATTGCTCCAG	TGGTTTTCCTATTGGAAGGCGG	124	FM863825
IFN-γ	Forward Reverse	AAGGGCTGTGATGTGTTTCTG TGTACTGAGCGGCATTACTCC	TTGATGGGCTGGATGACTTTAGGA	68	NM_001124620
IL-4/13a	Forward Reverse	ATCCTTCTCCTCTCTGTTGC GAGTGTGTGTGTATTGTCCTG	CGCACCGGCAGCATAGAAGT	139	AB574337
IgM	Forward Reverse	CTTGGCTTGTTGACGATGAG GGCTAGTGGTGTTGAATTGG	TGGAGAGAACGAGCAGTTCAGCA	72	S63348
IgT	Forward Reverse	AGCACCAGGGTGAAACCA GCGGTGGGTTCAGAGTCA	AGCAAGACGACCTCCAAAACAGAAC	73	AY870265
Y. ruckeri 16S	Forward Reverse	GCGAGGAGGAAGGGTTAAGTG GTTAGCCGGTGCTTCTTCTG	AATAGCACTGAACATTGACGTTACTCG	70	X75275
ELF-α	Forward Reverse	ACCCTCCTCTTGGTCGTTTC TGATGACACCAACAGCAACA	GCTGTGCGTGACATGAGGCA	63	AF498320

TABLE 3 Scoring set for microscopic evaluation of spleen lesions[a]

Parameter	0 (normal/minimal changes)	I (mild changes)	II (moderate changes)	III (severe changes)
Damage				
Capsular damage	NE	Sparse degenerative changes ± thickened BM	Regional damage with capsular disruption	Widespread damage with capsular disruption
Necrosis	NE	Focal/small scattered	Focally extensive to multifocal	Multifocal coalescing to diffuse
Reactive changes with negative impact				
Red pulp hyperplasia	Sinusoidal congestion ± sparse congested BVs	Frequent accumulated RBCs ± sparse congested BVs	Widely accumulated RBCs + sparse congested BVs	Dense/diffuse accumulated RBCs + sparse congested BVs
Cystic/clear space in parenchyma	NE	Indistinct/scattered small vacuoles	Noticeable small clear vacuoles	Clear empty spaces with eosinophilic coagulum + RBCs
Susceptibility—bacterial masses/colonies	NE	Frequent in ellipsoids ± sparse in sinusoids/parenchyma	Numerous in ellipsoids ± frequent in sinusoids/parenchyma	Extensive in ellipsoids + sinusoids/parenchyma
Protective cellular response				
Stromal cell hyperplasia & hypertrophy	Normal presence of cells	Frequent hyperplasia giving lobular appearance	Hyperplasia and hypertrophy ± cellular transformation	Extensive hyperplasia and hypertrophy + no evidence of lymphoid cells
Eosinophilic intercellular matrix		Frequent near many ellipsoids & sparse in stromal interstitium	Extensive around all ellipsoids and frequent in interstitium	Extensive in all portions of parenchyma
Active MΦ cells	Sparse near ellipsoids ± scattered in interstitium	Frequent near some ellipsoids + interstitium	Numerous near many ellipsoids + widely in interstitium	Extensive in all portions of parenchyma

[a] NE, none evident; BM, basement membrane; BVs, blood vessels; RBC, red blood cells; MΦ, macrophage.

groups but significant differences ($P < 0.05$) were noted between sham-challenged fish and all infected groups at day 14 p.i. It is noteworthy that generally higher levels of antibody reactivity were noted in all groups at 8 m.p.v. than at 4 m.p.v.

Gene expression. (i) Complement factors (C3 and C5). Following infection, liver tissues exhibited significant upregulation of genes encoding C3 and C5 at days 3 and 7 p.i. in vaccinated fish (Table 8). At day 7 p.i., nonimmunized *Y. ruckeri*-

TABLE 4 Scoring set for microscopic evaluation of head kidney lesion[a]

Parameter	0 (normal/minimal changes)	I (mild changes)	II (moderate changes)	III (severe changes)
Damage				
Sinusoidal/lobular disorganization	No changes	Mild disorganization	Marked disorganization	Severe disorganization
Hemorrhages	Sparse	Frequent	Widespread	Dense
Necrosis	NE	Focal/small scattered	Focally extensive to multifocal	Multifocal coalescing to diffuse
Reactive changes with negative impact				
Sinusoidal congestion	Scattered	Sparse to frequent	Numerous	Dense
Cellular depletion	NE	Indistinct/occasional	Evident	Prominent
Degree of cellularity and heterogeneity	Normal and regular	Mild loss of heterogeneity	Marked loss of heterogeneity ± loss of cellular parenchyma	Single-cell-type population + loss of cellular parenchyma
Susceptibility—bacterial masses/colonies	NE	Scattered	Frequent	Numerous and extensive
Protective cellular response				
MM cell aggregates	Frequent	Numerous	Extensive	More extensive and dense
Reticular cell content	Normal as near sinusoids	Mild increase	Notable increase ± edematous changes	Prominently present as meshwork-like parenchyma

[a] NE, not evident.

TABLE 5 Criteria for semiquantitative evaluation of CD8α cell density/distribution in spleen and head kidney

Immune parameter and score	Criterion
CD8α cell density/distribution	
0	No positive cells
0.5	Occasional presence of positive cells
1	Low/scattered presence of positive cells
2	Moderate/common presence of positive cells
3	Numerous positive cells
4	Extensive presence of positive cells throughout the section
CD8α staining intensity	
0	Negative/no staining
1	Yellow-to-brown staining of cells
2	Light-brown staining of cells
3	Brown staining of cells
4	Dark-brown staining of cells

infected fish also showed activation of the C3 gene. The expression of this gene in the spleen was low at all time points, but the C5 gene ended up being significantly upregulated in the AquaVac RELERA-vaccinated fish (day 7 p.i.). In head kidney, the C3 and C5 genes became highly upregulated on days 3 and 7 p.i. in AquaVac RELERA-vaccinated fish. Nonimmunized infected fish showed high expression of C5 genes on day 3 p.i.

TABLE 7 Scoring criteria used for *Y. ruckeri* antigen distribution in various organs studied

Score	Criterion
0	No staining observed (negative)
1	Few in number and focal in distribution (mild)
2	Few in number and multifocal in distribution (mild to moderate)
4	Several in number and multifocal in distribution (moderate)
5	Several in number and diffuse in distribution (moderate to severe)
6	Numerous with multifocal distribution (severe)
7	Numerous with diffuse distribution (very severe)

(ii) Acute-phase proteins and antimicrobial peptides (SAA, precerebellin, and cathelicidin 2). The expression patterns for the serum amyloid A (SAA) gene were very similar in liver, spleen, and head kidney, exhibiting significant upregulation in spleen and head kidney on day 3 p.i. in all infected groups (Table 8). This was followed by a further rise on day 7 p.i., with a noteworthy level of expression in liver tissues of nonimmunized *Y. ruckeri*-infected fish. The precerebellin gene in head kidney and spleen was highly expressed on day 3 p.i. in all infected groups, followed by decreased expression on day 7 p.i. Cathelicidin 2 (CATH 2) gene expression was relatively similar in all three organs, characterized by marked and significant upregulation on day 3 p.i., with the maximum level of expression in nonimmunized *Y. ruckeri*-infected fish and a minimal level of expression in AquaVac RELERA-vaccinated fish.

(iii) Lysozyme gene. All organs exhibited significant and high

TABLE 6 Criteria for semiquantitative evaluation of IgM cell density/distribution in spleen and head kidney

Organ	Immune parameter and score	Criterion
Spleen	IgM cell density/distribution	
	0	No positive cells
	0.5	Occasional & scattered cells/very few isolated aggregates of cells
	1	Few & scattered cells ± few small aggregates of cells in some places
	2	Several & scattered cells ± several aggregates of cells around BVs & other places
	3	Several & scattered cells + numerous aggregates of cells around BVs & other places
	4	Closely distributed cells + numerous aggregates of cells around the BVs & other places
	IgM secretory form	
	0	No secretory form observed
	1	Scattered presence
	2	Multifocal presence
	3	Wide presence
	4	Dense and nearly diffuse
Head kidney	IgM cell density/distribution	
	0	No positive cells
	0.5	Occasional & few positive cells
	1	Several & scattered positive cells ± few small aggregates of cells
	2	Evenly distributed positive cells or several-to-many thickly confined cells at some places
	3	Widely & evenly distributed positive cells + several small-to-large aggregates of cells
	4	Extensively present throughout the section
	IgM secretory form	
	0	No secretory form observed
	1	Scattered presence
	2	Thin multifocal reticulum-like intersinusoidal-space staining
	3	Large multifocal bridging-like intersinusoidal-space staining
	4	Large irregular bridging-like intersinusoidal-space staining involving whole sections

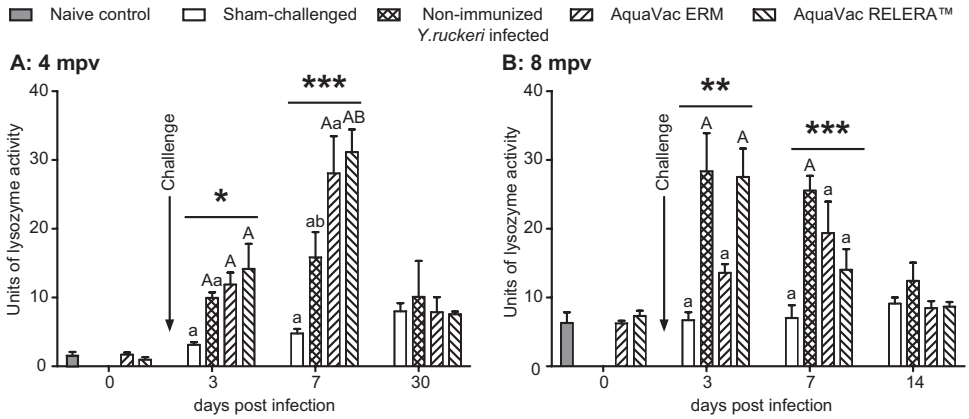

FIG 1 Serum lysozyme activity in fish following infection at 4 or 8 m.p.v. Each data bar represents the mean + SEM for 5 fish from the indicated group. Asterisks indicate significant differences among groups at the particular time point as analyzed by one-way ANOVA (*, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$). Upper- and lowercase letters indicate significant differences with respect to the sham-challenged group; same-case letters indicate that there is no significant difference between groups as determined by Tukey's multiple comparison tests.

levels of upregulation of the lysozyme gene on day 3 or 7 p.i., with the highest level of expression in AquaVac RELERA-vaccinated fish on the latter day (Table 8).

(iv) Cytokine genes associated with innate response (IL-1β, IL-6, TNF-α, and IL-10). The interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF-α) genes in spleen and head kidneys of nonimmunized *Y. ruckeri*-infected fish reacted especially significantly to infection at day 3 p.i., whereas the reactions in vaccinated fish were moderate (Table 9). Comparatively lower levels of mRNA transcripts for IL-1β and TNF-α genes were noted in liver of all infected groups. The IL-6 gene from this category was highly expressed in spleen on day 3 p.i. in nonimmunized *Y. ruckeri*-infected fish but was generally weakly activated in vaccinated groups. The IL-10 expression level was high in head kidney of all infected groups, showing a maximum in nonimmunized *Y. ruckeri*-infected fish at day 3 p.i.

(v) Cytokine genes associated with adaptive responses (IFN-γ and IL-4/13a). Both spleen and head kidney showed significant upregulation of the gamma interferon (IFN-γ) gene on day 3 p.i. in all infected groups, with high fold expression in nonimmunized infected fish and moderate activation in AquaVac RELERA-vaccinated fish (Table 9). The expression of the IL-4/13a gene was increased in liver of all vaccinated fish at this time point, but considerable variations were noted in other organs.

(vi) T cell marker genes (CD4 and CD8). The CD4 gene was upregulated more than 200-fold on day 3 p.i. in liver of nonimmunized *Y. ruckeri*-infected trout. The CD8 gene was not regulated in any fish at days 3 and 7 p.i. (Table 10).

(vii) T helper cell transcription factors (GATA-3 and T-bet). The GATA-3 gene was slightly upregulated in liver of nonimmunized *Y. ruckeri*-exposed fish but not in spleen and head kidney (Table 10). In contrast, T-bet gene expression in these organs was significantly downregulated in infected groups at day 3 p.i.

(viii) Immunoglobulin genes (IgM and IgT). Liver tissue exhibited significant upregulation of the IgM gene on day 7 p.i. in vaccinated fish (Table 10). The IgT gene was upregulated in vac-

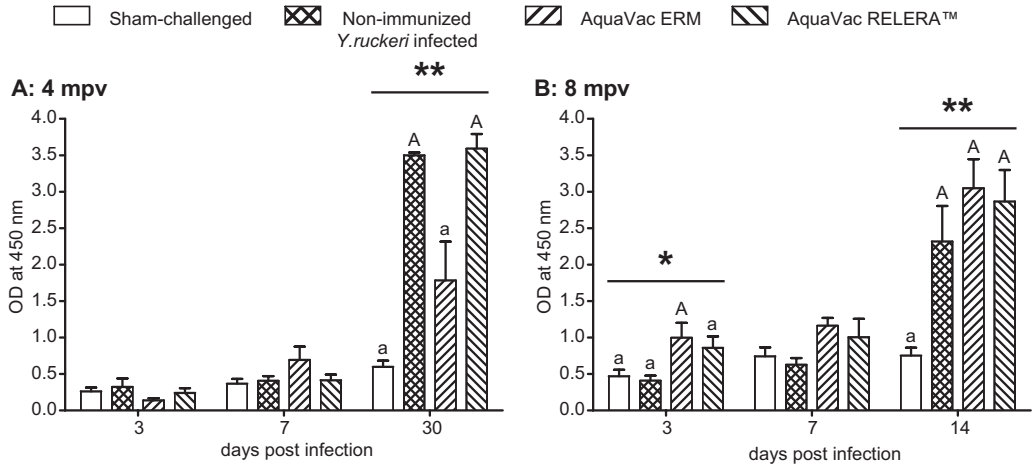

FIG 2 Serum antibody levels detected by ELISA in fish infected at 4 or 8 m.p.v. Each data bar represents the mean + SEM for 5 fish from the indicated group. Asterisks indicate significant differences among groups at the particular time point as analyzed by one-way ANOVA (*, $P = 0.05$; **, $P = 0.01$). Upper- and lowercase letters indicate significant differences with respect to the sham-challenged group; same-case letters indicate that there is no significant difference between groups as determined by Tukey's multiple comparison tests.

TABLE 8 Expression levels of immune genes regulating innate immunity determined by real-time quantitative PCR

Gene, organ	Mean expression (fold change ±SE) in indicated group at indicated no. of days p.i.[a]										
	Naive control	Sham challenged		Nonimmunized infected		AquaVac ERM vaccinated			AquaVac RELERA vaccinated		
	(0)	3	7	3	7	0	3	7	0	3	7
C3											
Liver	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	**3.9 ± 0.5*****	1.3 ± 0.1	**3.1 ± 0.5**** A	**5.8 ± 1.9***	1.27 ± 0.5	**5.7 ± 1.0**** a	**8.5 ± 1.5****
Spleen	1.0 ± 0.0	NV	NV	NV	NV	3.6 ± 2.0	NV	NV	2.2 ± 0.4	NV	NV
Head kidney	1.5 ± 0.5	1.0 ± 0.0	1.0 ± 0.1	1.3 ± 0.8	1.4 ± 0.1	3.2 ± 0.2	1.2 ± 0.3	1.9 ± 1.4 A	1.0 ± 0.4	11.2 ± 3.1	**4.9 ± 2.2** a
C5											
Liver	1.2 ± 0.3	1.0 ± 0.1	1.2 ± 0.3	1.3 ± 0.2	2.4 ± 0.6	1.6 ± 0.1	2.0 ± 0.4	**3.7 ± 0.8***	1.0 ± 0.2	2.0 ± 0.4*	**4.3 ± 0.5****
Spleen	1.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.2	1.5 ± 0.6	8.3 ± 3.0	2.7 ± 0.1	1.4 ± 0.0 A	1.4 ± 0.3 A	5.3 ± 0.0	**3.6 ± 0.0** a	**7.0 ± 2.4** a
Head kidney	1.1 ± 0.2	1.3 ± 0.4	1.1 ± 0.3	9.9 ± 7.1	1.1 ± 0.3	3.3 ± 0.1	2.1 ± 0.4 A	1.5 ± 0.3 A	1.6 ± 0.4	**16.8 ± 4.7** a	**10.3 ± 4.9** a
SAA											
Liver	2.9 ± 1.2	2.7 ± 1.6	2.7 ± 1.6	1,260 ± 627	**2,394 ± 825***	7.6 ± 3.6	999 ± 678	1,414 ± 1,050	1.9 ± 0.8	1,015 ± 551	6,202 ± 4653
Spleen	1.0 ± 0.1	1.9 ± 0.8	1.1 ± 0.2	**647 ± 156****	834 ± 451	1.6 ± 0.5	**937 ± 253****	236 ± 129	5.8 ± 2.9	751 ± 505	**496 ± 210***
Head kidney	1.1 ± 0.2	1.1 ± 0.2	1.2 ± 0.2	**910 ± 203****	**272 ± 98.3***	2.0 ± 0.6	**842 ± 276***	190 ± 93.7	3.5 ± 0.9	**623 ± 253***	1,086 ± 667
Precerebellin											
Liver	1.0 ± 0.1	1.1 ± 0.2	1.1 ± 0.3	**6.1 ± 1.1****	**23.9 ± 7.7***	2.3 ± 0.7	**5.8 ± 0.6*****	**16.8 ± 5.6***	1.3 ± 0.3	**5.5 ± 0.6*****	**30.1 ± 7.0*****
Spleen	1.2 ± 0.2	1.8 ± 0.6	1.4 ± 0.5	**3,646 ± 134***	325 ± 219	7.5 ± 5.1	**2,030 ± 376****	171.6 ± 96.7	4.3 ± 1.4	**739 ± 489** a	88.4 ± 45.4
Head kidney	1.0 ± 0.1	1.4 ± 0.4	1.4 ± 0.3	**159 ± 30****	**55.3 ± 23.8***	1.6 ± 0.7	**154 ± 9.8*****	39.1 ± 20.5	1.3 ± 0.4	**96.5 ± 19****	72.3 ± 41.2
CATH2											
Liver	3.7 ± 1.7	1.0 ± 0.2	1.3 ± 0.6	3,109 ± 985	122.6 ± 81.7	2.7 ± 0.9 A	1,344 ± 856	117 ± 76.3	**3.0±0.14** a	638 ± 475	165 ± 68.2
Spleen	1.5 ± 0.4	2.2 ± 1.1	1.5 ± 0.5	**1,061 ± 281****	294 ± 226	11.2 ± 8.6	**1,799 ± 639***	31.1 ± 16.4	10.4 ± 6.7	**518 ± 220***	**148 ± 56.7***
Head kidney	1.2 ± 0.3	1.1 ± 0.2	1.1 ± 0.3	**1,311 ± 425***	79.2 ± 38.0	2.3 ± 0.8	**879 ± 179****	208.4 ± 150	3.1 ± 1.4	481 ± 300	174 ± 67.6
Lysozyme											
Liver	1.3 ± 0.4	1.1 ± 0.2	1.2 ± 0.4	**3.1 ± 0.7***	**15.0 ± 2.6*****	1.4 ± 0.4	**6.0 ± 1.8***	**25.1 ± 8.0***	1.2 ± 0.2	**5.8 ± 0.3*****	**39.5 ± 9.8*****
Spleen	1.0 ± 0.1	1.5 ± 0.7	2.2 ± 0.8	**6.7 ± 1.5***	**9.8 ± 2.9***	9.2 ± 6.6	**7.9 ± 1.5****	10.9 ± 4.3	2.8 ± 0.6	26.3 ± 20.9	**6.0 ± 1.6***
Head kidney	1.2 ± 0.3	1.9 ± 1.0	1.2 ± 0.4	1.2 ± 0.9	**12.1 ± 3.0****	1.2 ± 0.8	3.7 ± 1.1	**7.7 ± 2.1***	1.6 ± 0.3	7.2 ± 2.4	**20.9 ± 6.5***

[a] Only >3-fold changes were considered. Significantly different values are shown in boldface and specified as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Differences noted between two vaccinated groups are indicated with A or a. Comparisons were made with regard to the naive control group before challenge and the sham-challenged group after challenge at each time point. Underscores show negative values. NV, no C_T value was obtained.

TABLE 9 Expression levels of pro-/anti-inflammatory cytokine genes determined by real-time quantitative PCR

Gene, organ	Mean expression (fold change ±SE) in indicated group at indicated no. of days p.i.[a]										
	Naive control	Sham challenged		Nonimmunized infected		AquaVac ERM vaccinated			AquaVac RELERA vaccinated		
	(0)	3	7	3	7	0	3	7	0	3	7
IL-1β											
Liver	1.8 ± 0.8	1.0 ± 0.2	1.0 ± 0.0	991 ± 291	6.5 ± 3.8	5.4 ± 0.0	326 ± 95.8	10.4 ± 7.8	1.5 ± 1.1	248 ± 154.8	10.0 ± 0.2
Spleen	1.2 ± 0.4	1.3 ± 0.3	1.0 ± 0.2	**1,322 ± 372****	**35.3 ± 15.8***	5.2 ± 0.0	**476 ± 136****	14.0 ± 8.7	7.1 ± 5.9	231 ± 116.6	**5.4 ± 1.4***
Head kidney	1.2 ± 0.3	1.1 ± 0.2	1.0 ± 0.1	**1,002 ± 278****	**7.5 ± 2.3***	**3.6 ± 0.0***	**54.6 ± 13.5****	10.8 ± 8.4	1.1 ± 0.2*	**29.1 ± 8.4****	5.2 ± 2.3
IL-6											
Liver	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV	NV
Spleen	1.5 ± 0.6	4.0 ± 2.4	1.2 ± 0.3	**9,055 ± 2,325****	8.7 ± 5.1	1.7 ± 0.2	822.3 ± 605.9	6.7 ± 3.4	1.1 ± 0.7	47.7 ± 20.4	1.9 ± 0.6
Head kidney	1.1 ± 0.3	1.0 ± 0.0	1.0 ± 0.2	1.2 ± 336	4.0 ± 1.8	2.3 ± 0.1	**23.8 ± 4.4** A	1.6 ± 1.0	3.1 ± 0.0	**6.9 ± 1.6** a	1.7 ± 0.5
TNF-α											
Liver	1.4 ± 0.5	1.0 ± 0.0	1.0 ± 0.0	15.4 ± 4.1	**11.3 ± 2.4***	1.6 ± 0.9	**10.0 ± 3.0** A	5.9 ± 2.4	1.7 ± 0.8	**3.5 ± 1.1a**	**10.3 ± 1.6***
Spleen	1.3 ± 0.4	1.3 ± 0.5	1.0 ± 0.2	**70.1 ± 23.4***	**9.7 ± 3.3***	2.5 ± 0.1	**52.0 ± 13.3****	2.6 ± 0.8	3.5 ± 2.8	22.8 ± 6.4	**2.0 ± 0.1****
Head kidney	1.3 ± 0.3	1.0 ± 0.0	1.0 ± 0.1	**19.7 ± 4.4****	1.4 ± 0.2	2.5 ± 0.0*	**4.6 ± 0.6*****	1.5 ± 0.2 A	1.6 ± 0.1	4.0 ± 0.6	1.2 ± 0.1 a
IL-10											
Liver	1.0 ± 0.1	NV	1.0 ± 0.1	NV	81.8 ± 64.1	1.6 ± 0.0	NV	26.3 ± 19.6	**2.3 ± 0.0***	NV	51.4 ± 35.6
Spleen	1.9 ± 1.2	1.0 ± 0.0	1.8 ± 1.0	156 ± 43.5	453.8 ± 382.7	1.1 ± 0.0	525 ± 217	29.8 ± 20.9	5.2 ± 3.8	377 ± 226	47.7 ± 22.0
Head kidney	1.5 ± 0.8	1.0 ± 0.1	1.0 ± 0.1	**1,525 ± 42***	95.8 ± 74.9	3.2 ± 0.0	231 ± 98.1	40.5 ± 34.0	3.6 ± 0.0	75.7 ± 37.0	70.4 ± 38.0
IFN-γ											
Liver	1.1 ± 0.2	1.4 ± 0.7	1.0 ± 0.2	54.5 ± 19.7	14.3 ± 8.7	2.6 ± 1.0	27.5 ± 7.1	29.6 ± 14.6	1.0 ± 0.1	20.2 ± 5.2	34.1 ± 14.4
Spleen	1.2 ± 0.3	1.2 ± 0.3	1.9 ± 0.8	**115 ± 49.4***	10.4 ± 5.9	1.1 ± 0.2	**21.5 ± 6.1****	1.8 ± 0.6	1.2 ± 0.1	12.6 ± 6.1	3.0 ± 0.5
Head kidney	1.2 ± 0.3	1.0 ± 0.1	1.4 ± 0.6	**45.8 ± 9.7****	**12.6 ± 3.8***	1.9 ± 0.7	**28.5 ± 7.5****	13.8 ± 10.5	1.5 ± 0.3	**21.0 ± 3.7*****	25.8 ± 17.8
IL-4/13a											
Liver	1.0 ± 0.0	1.1 ± 0.2	1.3 ± 0.39	2.7 ± 0.6*	1.8 ± 0.5	1.5 ± 0.1	**3.0 ± 0.2*****	1.8 ± 0.4	1.1 ± 0.1	**3.2 ± 0.6***	2.3 ± 0.6
Spleen	1.2 ± 0.3	1.7 ± 0.6	1.4 ± 0.5	1.5 ± 0.5	1.4 ± 0.3	1.0 ± 0.2	2.3 ± 0.2	1.3 ± 0.3	2.1 ± 1.0	3.8 ± 1.3	1.1 ± 0.4
Head kidney	1.1 ± 0.2	1.00.0	1.1 ± 0.3	1.2 ± 0.2	1.9 ± 0.4	1.6 ± 0.1	1.2 ± 0.1	2.1 ± 1.0	1.4 ± 0.1	1.0 ± 0.1	2.9 ± 0.7

[a] Only >3-fold changes were considered. Significantly different values are shown in boldface and specified as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Differences noted between two vaccinated groups are indicated with A or a. Comparisons were made with regard to the naive control group before challenge and the sham-challenged group after challenge at each time point. Underscores show negative values. NV, no C_T value was obtained.

cinated fish at day 7, whereas it was significantly downregulated in nonimmunized *Y. ruckeri*-infected fish (liver and spleen) at day 3 p.i.

Detection of *Y. ruckeri* 16S rRNA transcripts. The infection levels in liver, spleen, and head kidney were also assessed by measuring bacterial 16S transcripts. At day 3 p.i., significantly lower levels of *Y. ruckeri* 16S transcript were observed in all vaccinated groups than in nonimmunized exposed groups (see Fig. 8). Despite the consistently lower levels of *Y. ruckeri* in AquaVac RELERA-vaccinated fish, no significant differences were noted between AquaVac RELERA vaccinates and AquaVac ERM vaccinates when analyzed tissue by tissue. However, following pooling of tissues across the groups, a significant difference ($P < 0.05$) was obtained between the two vaccinated groups at day 3 p.i.

Pathological changes in spleen and head kidney. Following stimulation (either vaccination or infection/injection), we noted certain cells with special morphologies in both spleen and head kidney. They appeared to originate from resident cells of the organ. In spleen, epithelioid/macrophage-like cells, lymphostromal cells (i.e., pleomorphic cells with polymorphic ability for both lymphoid and stromal cells), and enlarged (Fig. 3A) eccentrically nucleated lymphoid cells with amphophilic cytoplasm were evident. The head kidney showed either enlarged lymphoid cells having a macrophage-like appearance (Fig. 3B) or medium-sized, reniform-to-cleaved nucleated lymphoid cells, besides putative monocytes with greyish vacuolated cytoplasm. In this study, epithelioid-like cells in spleen are considered to be activated macrophage cells.

(i) Naive control group (unchallenged). Fish from the naive control group were found to be free from major cellular changes.

(ii) Sham-challenged (PBS-injected) group. ***(a)*** **Spleen.** No major cellular changes were detected in spleen in the sham-challenged group, except for melanomacrophage cell (MM cell) hyperplasia (Fig. 4, panel 1A).

(b) **Head kidney.** A minor amount of interrenal tissue around veins and wide areas of MM cells/aggregates in the parenchyma characterized the changes in head kidney in the sham-challenged group during the first week (Fig. 5, panel 1A). An extensive presence of MM cells and increased numbers of medium-sized lymphoid cells (reniform to cleaved nucleated) and few monocytes in hemopoietic cell islands were noticed at later stages (i.e., 21 to 30 days postchallenge).

(iii) Nonimmunized *Y. ruckeri*-infected group. ***(a)*** **Spleen.** The changes in spleen in the nonimmunized *Y. ruckeri*-infected group did not differ much between fish observed at either 4 or 8 m.p.v. At day 3 p.i., the changes comprised the presence of eosinophilic necrotic cuffs around arterioles/ellipsoids, depletion of resident lymphoid and MM cells/aggregates, and loss of intercellular matrix (Fig. 4, panel 2A). The presence of several macrophages, macrophage-like transformation of several hypertrophied sinusoidal cells, and hypertrophy of stromal cell and sinusoidal cells, besides a few floral-like arrangements of red blood cells (RBCs) near necrotic cuffs, were evident. On day 7 p.i., multifocal occurrences of macrophage-like cells (both near ellipsoids and in interstitium) were observed. Evidence of eosinophilic amorphous

TABLE 10 Expression levels of immune genes regulating adaptive immunity determined by real-time quantitative PCR

Gene and organ	Mean expression (fold change ±SE) in indicated group at indicated no. of days p.i.[a]										
	Naive control (0)	Sham challenged		Nonimmunized infected		AquaVac ERM vaccinated			AquaVac RELERA vaccinated		
		3	7	3	7	0	3	7	0	3	7
CD4											
Liver	1.9 ± 0.9	1.4 ± 0.4	1.0 ± 0.1	**294 ± 66.6****	7.4 ± 3.9	2.0 ± 0.8	182 ± 97.7	10.6 ± 7.8	1.4 ± 0.4	253 ± 179.9	17.6 ± 10.5
Spleen	1.1 ± 0.2	1.3 ± 0.4	1.1 ± 0.2	2.0 ± 0.3	2.6 ± 0.6*	1.5 ± 0.4	1.7 ± 0.5	1.9 ± 0.0* A	1.4 ± 0.5	2.1 ± 0.4	1.3 ± 0.3 a
Head kidney	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.3	1.1 ± 0.2	1.5 ± 0.3	1.1 ± 0.2	1.1 ± 0.0	1.0 ± 0.4	1.0 ± 0.1	1.0 ± 0.2	2.4 ± 0.8
CD8											
Liver	1.9 ± 1.1	2.1 ± 0.8	1.2 ± 0.3	2.0 ± 0.7	2.5 ± 1.0	1.5 ± 0.5	1.3 ± 0.3	1.1 ± 0.6	2.8 ± 1.1	2.6 ± 0.7	2.1 ± 0.5
Spleen	1.3 ± 0.4	2.1 ± 1.0	1.7 ± 0.8	4.5 ± 0.0	1.8 ± 0.3	1.4 ± 0.5	2.0 ± 0.2	1.5 ± 0.1	4.4 ± 2.3	2.0 ± 0.1	2.1 ± 0.8
Head kidney	1.1 ± 0.2	1.2 ± 0.4	1.1 ± 0.2	2.4 ± 0.0	1.0 ± 0.2	1.4 ± 0.2	1.7 ± 0.1	1.3 ± 0.3	1.7 ± 0.1	1.0 ± 0.2	1.8 ± 0.5
T-bet											
Liver	1.1 ± 0.2	1.4 ± 0.6	2.2 ± 0.8	1.4 ± 0.3	3.8 ± 1.5	1.0 ± 0.1	2.4 ± 0.7	5.7 ± 1.6	1.1 ± 0.2	2.1 ± 1.2	18.9 ± 6.4
Spleen	1.2 ± 0.2	1.2 ± 0.3	1.2 ± 0.4	2.0 ± 0.1*	1.3 ± 0.0	2.1 ± 0.7	1.9 ± 0.0*	**3.2 ± 0.0***	1.9 ± 0.4	2.8 ± 0.0*	1.5 ± 0.1
Head kidney	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	3.1 ± 0.0***	1.1 ± 0.2	1.3 ± 0.1	**4.4 ± 0.0*****	1.4 ± 0.2	1.1 ± 0.1	**3.7 ± 0.0*****	1.4 ± 0.5
GATA-3											
Liver	1.3 ± 0.4	1.3 ± 0.5	1.1 ± 0.2	**4.7 ± 0.9***	14.8 ± 9.0	1.0 ± 0.2	1.8 ± 0.4	2.4 ± 0.9	1.2 ± 0.5	5.6 ± 2.1	**5.8 ± 1.8***
Spleen	1.3 ± 0.3	1.4 ± 0.4	1.9 ± 0.8	1.6 ± 0.6	1.5 ± 0.3	1.1 ± 0.2	1.1 ± 0.4	**6.0 ± 0.0** A	1.3 ± 0.4	1.0 ± 0.2	1.0 ± 0.3 a
Head kidney	1.1 ± 0.3	1.0 ± 0.1	1.5 ± 0.6	1.1 ± 0.2	1.7 ± 0.5	1.5 ± 0.1	1.2 ± 0.1	1.2 ± 0.6	1.5 ± 0.0	1.1 ± 0.1	3.4 ± 1.0
IgM											
Liver	1.0 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	2.9 ± 0.9	2.6 ± 0.9	1.4 ± 0.5	1.5 ± 0.2	2.9 ± 0.6*	1.5 ± 0.3	2.0 ± 0.4	**6.5 ± 2.6***
Spleen	1.1 ± 0.2	2.1 ± 1.2	1.1 ± 0.2	1.3 ± 0.3	1.2 ± 0.0	1.4 ± 0.3	1.2 ± 0.2	1.7 ± 0.1*	1.7 ± 0.2	1.0 ± 0.2	1.0 ± 0.3
Head kidney	1.1 ± 0.2	1.2 ± 0.4	1.1 ± 0.1	1.0 ± 0.1	1.7 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.9 ± 0.3*	1.3 ± 0.0	1.0 ± 0.2	2.6 ± 0.7
IgT											
Liver	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	**4.2 ± 0.0****	1.6 ± 0.7	1.0 ± 0.2	1.3 ± 0.2 A	1.2 ± 0.2	3.0 ± 1.4	2.0 ± 0.5 a	**4.2 ± 1.4***
Spleen	1.2 ± 0.2	1.4 ± 0.4	1.5 ± 0.5	**7.0 ± 0.0***	2.0 ± 0.1	2.9 ± 0.1*	1.0 ± 0.3	3.2 ± 0.0	1.6 ± 0.8	1.1 ± 0.4	1.4 ± 0.2
Head kidney	1.1 ± 0.2	1.1 ± 0.3	1.3 ± 0.3	1.4 ± 0.1	1.9 ± 0.4	2.1 ± 0.1	1.0 ± 0.2	3.0 ± 1.5	1.1 ± 0.2	1.0 ± 0.2	3.6 ± 1.4

[a] Only >3-fold changes were considered. Significantly different values are shown in boldface and specified as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Differences noted between two vaccinated groups are indicated with A or a. Comparisons were made with regard to the naive control group before challenge and the sham-challenged group after challenge at each time point. Underscores show negative values. NV, no C_T value was obtained.

intercellular matrix and many thin MM cell aggregates were other prominent findings. By day 21 p.i., lymphoid and MM cell reappearance began gradually, along with detection of several lymphostromal-like cells and many macrophages near ellipsoids. At day 30 p.i., further widespread repopulation of the parenchyma with both MM and lymphoid cells was evident.

***(b)* Head kidney.** At day 3 p.i., the main changes in head kidney in the nonimmunized *Y. ruckeri*-infected group were lymphoid cell transformation to macrophage-like cells and further appearance of a meshwork-like parenchyma (Fig. 5, panel 2A). Other notable changes were sinusoidal cell hypertrophy and sparse presence of MM cells/aggregates and interrenal cells around veins. At day 7 p.i., medium-sized (reniform-to-cleaved nucleated) lymphoid cells became prominent and were followed by a few vacuolated monocytes and very few granuloid cells and mitotic cells (putative erythroid cells). Between days 21 and 30 p.i., cellular changes similar to those seen at day 7 p.i. were generally observed but with reappearance of interrenal cells and moderate-to-extensive presence of MM cells/ag-

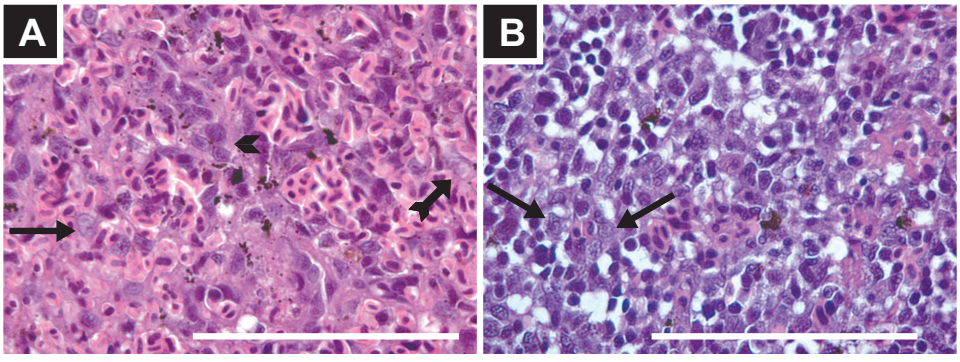

FIG 3 (A) Spleen tissue, showing transformation of lymphoid cells into stromal-like cells (arrowhead), stromal cells into macrophage-like cells (arrow), and sinusoidal cells into macrophage-like cells (feather-end arrow) at day 7 postchallenge. (B) Head kidney tissue, showing transformation of lymphoid cells into macrophage-like cells (arrows) at day 3 postchallenge. H&E magnification, ×40. Scale bars = 100 μm.

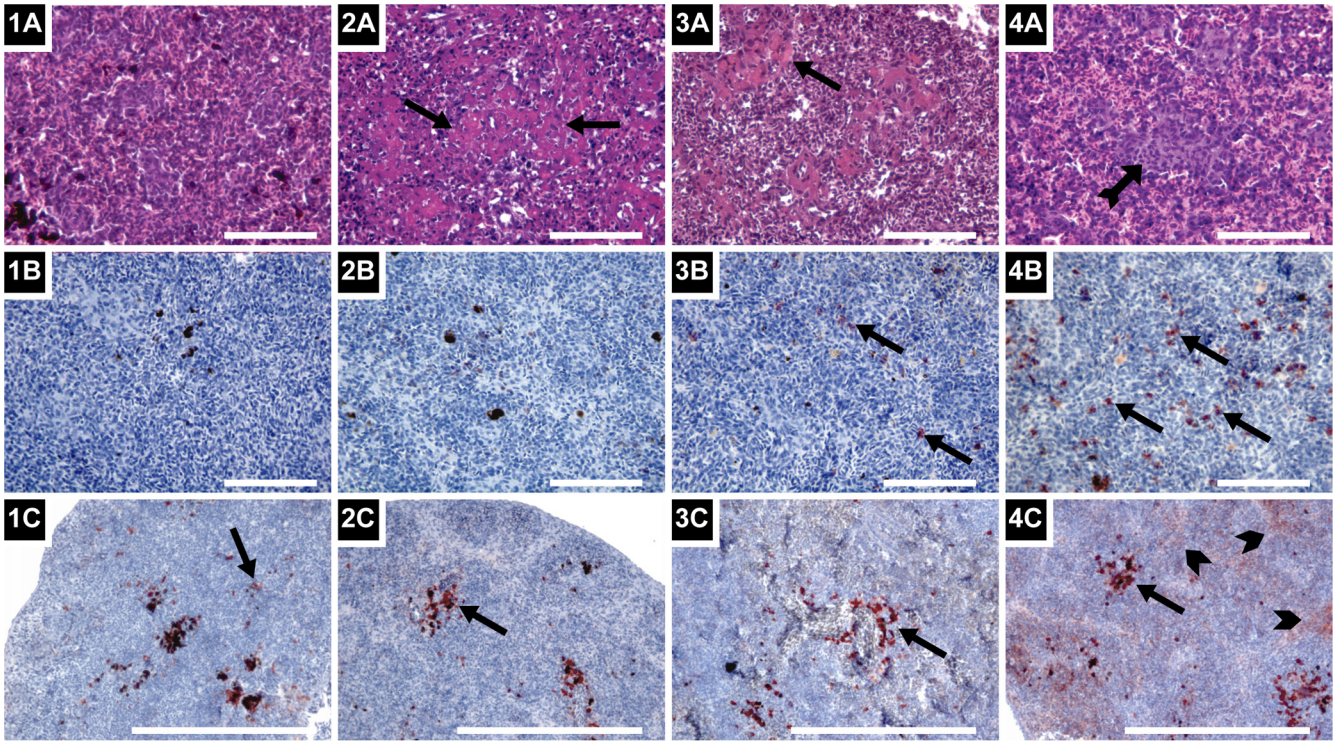

FIG 4 Spleen samples from sham-challenged (1st column), *Y. ruckeri*-infected (2nd column), AquaVac ERM-vaccinated (3rd column), and AquaVac RELERA-vaccinated (4th column) fish infected at 4 m.p.v. (Row A) Collected on day 3 p.i., stained with H&E, ×20 magnification, arrow indicates necrotic parenchyma, feather-end arrow (4th column) indicates stromal cell hyperplasia. (Row B) Collected on day 30 p.i., stained for CD8α cells (IHC), ×20 magnification, arrows indicate positive cells. (Row C) Collected on day 30 p.i., stained for IgM cells (IHC), ×10 magnification, arrows indicate positive cells, arrowheads indicate IgM secretory form. Scale bars = 100 μm.

gregates. The changes seen at 4 m.p.v. corresponded more or less to those seen at 8 m.p.v., with early onset and quick waning.

(iv) AquaVac ERM-vaccinated group. ***(a)*** **Spleen.** The changes seen in spleen in the AquaVac ERM-vaccinated group before infection at 4 m.p.v. included the presence of hypertrophic stromal cells and macrophages near ellipsoids, in addition to moderate presence of an eosinophilic amorphous intercellular matrix. Following infection (i.e., at day 3 p.i.), numerous lymphostromal-like cells and their subsequent transformation to macrophages and epithelioid-like cells were observed (both near ellipsoids and in interstitium). Prominent sinusoidal cell hypertrophy, loss of MM cells/aggregates, multifocal necrosis, and depletion of eosinophilic amorphous intercellular matrix were also noted (Fig. 4, panel 3A). At day 7 p.i., the eosinophilic amorphous intercellular matrix had widened significantly, with disappearance of macrophages and epithelioid-like cells from the interstitium. Between days 21 and 30 p.i., dense populations of lymphoid cells with a few lymphostromal-like cells around ellipsoids were evident. Wide and thick aggregates of MM cells across the parenchyma were very common. At 8 m.p.v., cellular changes more-or-less similar to those at day 3 p.i. prevailed, with no loss of MM cells/aggregates and eosinophilic amorphous intercellular matrix. The regular cellular structure was regained by day 14 p.i.

(b) **Head kidney.** Before infection (at 4 m.p.v.), no major cellular changes were noticed in head kidney in the AquaVac ERM-vaccinated group. Following infection (from day 3 to 7 p.i.), the early cellular changes were more-or-less similar to the findings in nonimmunized fish but with reduced transformation of macrophage-like cells to fibrous reticular-like cells. In addition, the numbers of single-cell necrotic foci were fewer (Fig. 5, panel 3A). Between days 21 and 30 p.i., reestablishment of the normal basic structure was observed, which included cellular heterogeneity and the reappearance of interrenal cells and MM cells. Almost-similar but less-severe changes were noticed at 8 m.p.v.

(v) AquaVac RELERA-vaccinated group. ***(a)*** **Spleen.** Prior to infection at 4 m.p.v., the cellular response noted in spleen in the AquaVac RELERA-vaccinated group was similar to that in AquaVac ERM vaccinates, with additional evidence of abundant lymphostromal-like cells. After infection (i.e., at day 3 p.i.), a marked increase of lymphostromal cell density was seen, along with sinusoidal cell hypertrophy (Fig. 4, panel 4A). However, only a few of the lymphostromal cells seemed to transform into macrophages and epithelioid-like cells. The eosinophilic amorphous intercellular matrix became dominated by eccentrically nucleated lymphoid cells (having amphophilic cytoplasm) and contained many small-sized aggregates of MM cells. At day 7 p.i., these changes were supplemented with transformation of hypertrophic sinusoidal cells to macrophage-like cells. From day 21 to day 30 p.i., the cellular changes corresponded closely to observations of spleen in AquaVac ERM-vaccinated fish. The changes at 8 m.p.v. were considerably less intense, and reestablishment of dense lymphoid cell populations was noted by day 7 p.i. and onwards.

(b) **Head kidney.** Before infection, the changes in head kidney in the AquaVac RELERA-vaccinated group were negligible, but following infection at 4 m.p.v. (i.e., after day 3 p.i.), prominent proliferation of medium-sized lymphoid cells with varied nuclear

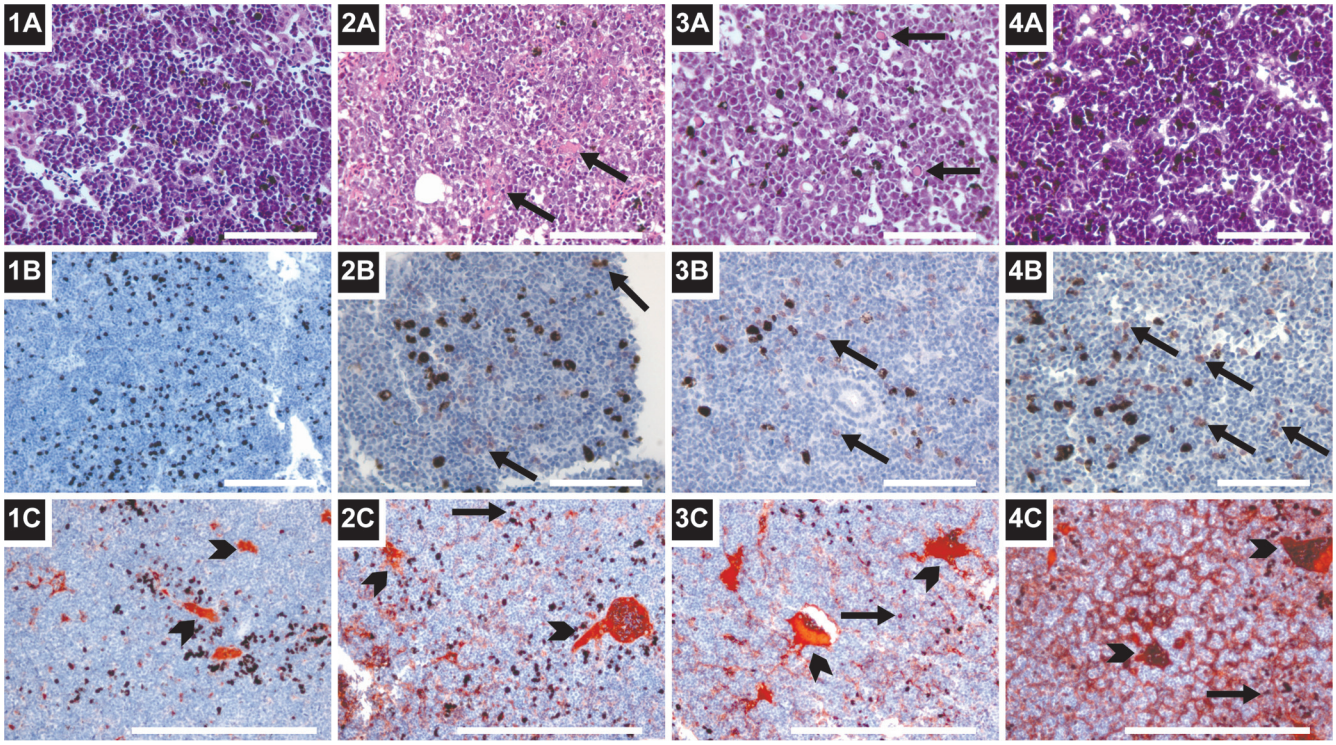

FIG 5 Head kidney samples from sham-challenged (1st column), *Y. ruckeri*-infected (2nd column), AquaVac ERM-vaccinated (3rd column), and AquaVac RELERA-vaccinated (4th column) fish infected at 4 m.p.v. (Row A) Collected on day 3 p.i., stained with H&E, ×20 magnification, arrow indicates necrotic parenchyma/cells. (Row B) Collected on day 30 p.i., stained for CD8α cells (IHC), ×20 magnification, arrows indicate positive cells. (Row C) Collected on day 30 p.i., stained for IgM cells (IHC), ×10 magnification, arrows indicate positive cells, arrowheads indicate IgM secretory form. Scale bars = 100 μm.

morphologies (i.e., reniform-to-cleaved pattern) and some monocytes/macrophages were noted. Aggregates of MM cells were widely distributed, and a moderate increase in the occurrence of stellate/spindle-shaped fibrous reticular cells across the parenchyma was seen (Fig. 5, panel 4A). In these relatively well-protected fish, normal interrenal cells reappeared around veins by day 3 p.i. By day 7 p.i., further propagation of stellate/spindle-shaped fibrous reticular cells was evident. Between days 21 and 30 p.i., no major cellular changes, apart from occasional sinusoidal cell hypertrophy, were noted. At 8 m.p.v., almost the same changes were noticed.

Immune cell distribution and density in spleen and head kidney. (i) CD8α$^+$ cells. In spleen and head kidney, CD8α cells (solitary or aggregated) were seen in low numbers mainly around blood vessels and often near MM cell aggregates. In the spleen, at 4 m.p.v., before infection challenge, the presence of CD8α cells was more-or-less similar in all groups investigated. Following infection (at day 3 p.i.), a transient decrease in CD8α cell density until day 7 was noted in all infected groups except for AquaVac RELERA vaccinates, which were unaffected. At day 30 p.i., increased densities of CD8α cells were noticed in all infected groups, but the highest increase was in AquaVac RELERA vaccinates (Fig. 4, row B). At 8 m.p.v., following infection (at day 3 p.i.), no clear change of CD8α$^+$ cell distribution was seen, but the number of CD8α$^+$ cells in nonimmunized *Y. ruckeri*-infected fish increased from day 7 p.i. In head kidney, CD8α cell distribution corresponded to the patterns in spleen at all time points (4 m.p.v. [Fig. 5, row B] and 8 m.p.v.).

(ii) IgM$^+$ cells. In spleen, the IgM-producing cells were seen as independent cells (cytoplasmic granulated cell) throughout the parenchyma and as aggregates of cells around the blood vessels in close proximity to MM aggregates. IgM in a secreted form was seen as light-orange- to rose-red-colored diffuse staining either in sinusoidal spaces or in the intercellular matrix and in the lumen of blood vessels. In head kidney, the distribution of IgM-positive cells and the secreted form of IgM corresponded to the occurrence in spleen tissue but had a more-distinct reticular style of staining in intersinusoidal spaces.

In spleen, prior to infection, the densities of IgM cells appeared similar in all groups investigated (at both 4 and 8 m.p.v.). After infection (at day 3 p.i., challenge at 4 m.p.v.), declines in IgM$^+$ cell density were noticed in nonimmunized *Y. ruckeri*-infected fish and AquaVac ERM vaccinates, whereas AquaVac RELERA-vaccinated fish showed no distinct changes. A slight increase in IgM cell density was noted in all infected groups from day 7 p.i. When challenged at 8 m.p.v., little or no decrease in IgM$^+$ cells was observed in vaccinated groups, while a decline in IgM cells was noted in *Y. ruckeri*-infected fish.

At 4 m.p.v., vaccinated groups revealed increased levels of the secreted form of IgM on day 3 p.i., slight decreases at day 7 p.i., and minor rises on day 30 p.i. (Fig. 4, row C). However, at 8 m.p.v., no major variation was noted in any group following challenge.

In head kidney, no clear change was seen at day 3 p.i., but at later time points (i.e., from day 7 p.i.), IgM cell densities increased in all groups. Corresponding variations were seen at 8 m.p.v. During the first week of infection (at both 4 and 8 m.p.v.), the occurrence of the secreted form of IgM was noted in all groups investigated. The highest level of secreted IgM was noted in AquaVac RELERA vaccinates at 4 m.p.v. (Fig. 5, row C).

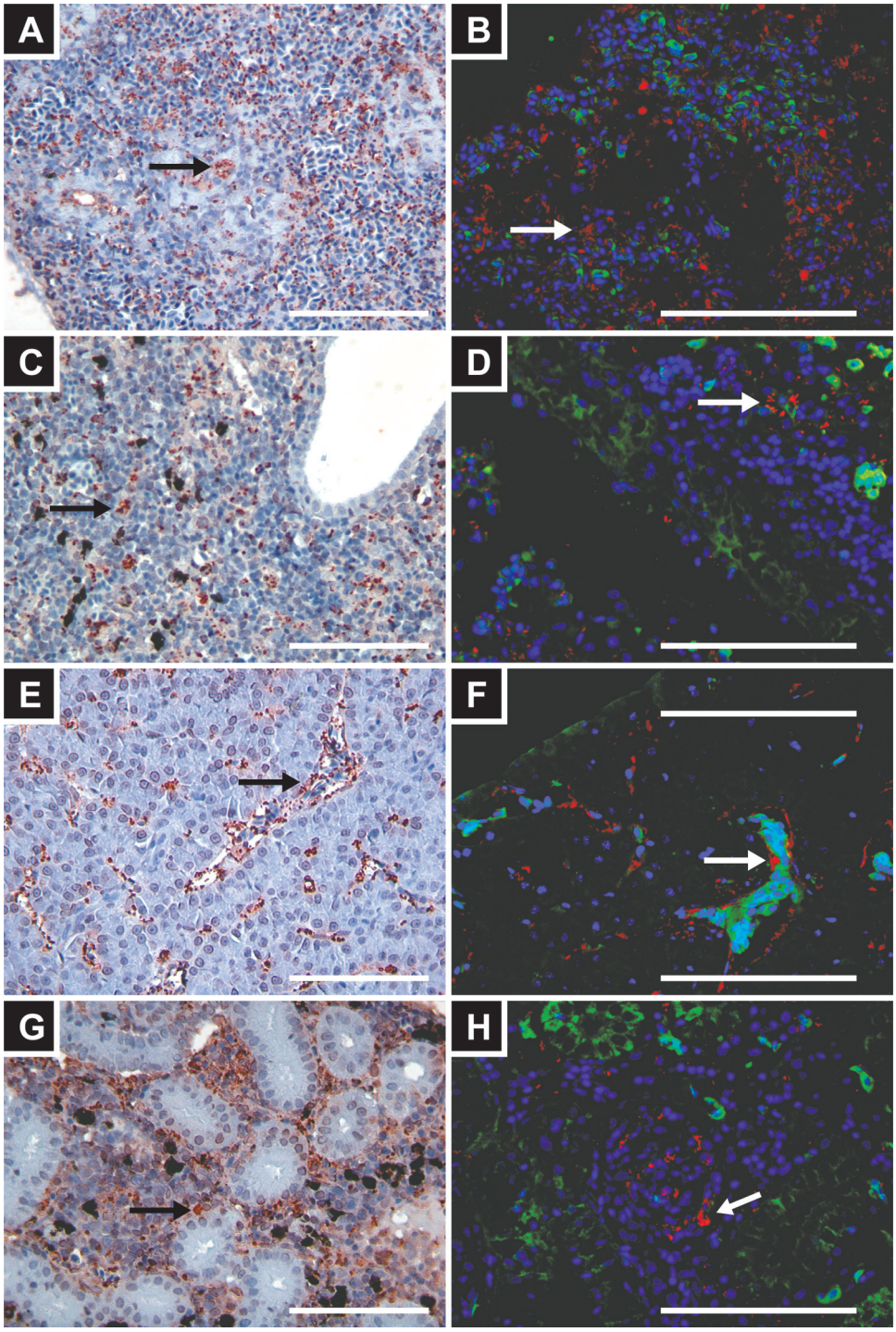

FIG 6 Representative tissue samples collected on day 3 p.i. from nonimmunized fish infected with *Y. ruckeri* pathogen at 4 m.p.v. The immunohistochemistry (IHC) staining was conducted using mouse monoclonal anti-*Y. ruckeri* antibody (clone 7F5/F6) and fluorescent *in situ* hybridization (FISH) using species-specific probe targeting 16S rRNA of *Y. ruckeri* (×200 magnification). Arrows in left-hand panels of the pairs indicate bacterial antigen, and arrows in right-hand panels indicate intact bacteria. No intact bacteria could be seen in heart tissue. (A and B) spleen; (C and D) head kidney; (E and F) liver; (G and H) posterior kidney; (I and J) intestine; (K and L) gills; (M and N) heart. Scale bars = 100 μm.

***Y. ruckeri* antigen distribution in various organs.** A notable presence of *Y. ruckeri* antigen was noted in the spleen of infected fish, whereas merely limited occurrence was seen in intestine and gills (Fig. 6). None of the sham-challenged fish showed any presence of *Y. ruckeri* antigen at any time point. Following bacterial exposure, the occurrence of *Y. ruckeri* antigen was significantly lower in AquaVac RELERA vaccinates than in AquaVac ERM-vaccinated fish and the nonimmunized *Y. ruckeri*-infected group (Fig. 7).

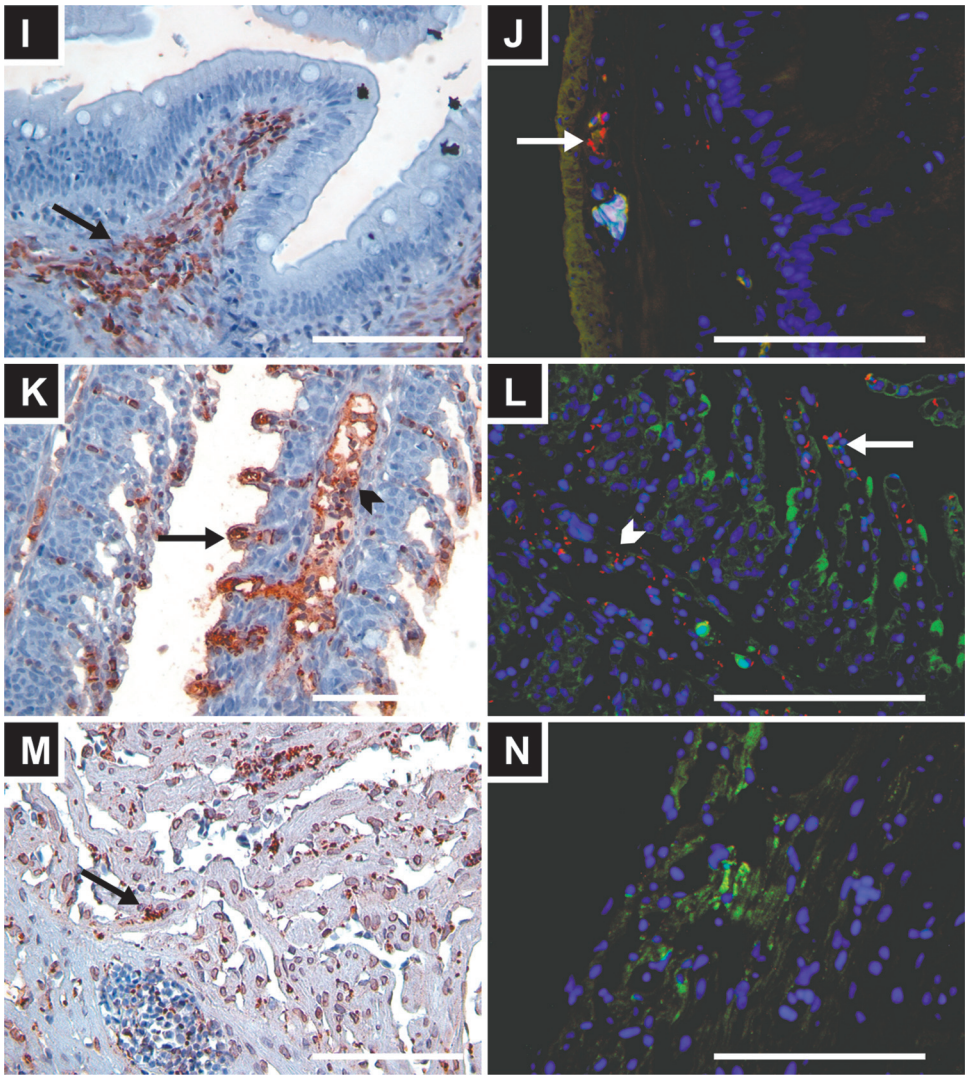

FIG 6 continued

FISH. No intact bacteria were detected in the AquaVac RELERA-vaccinated fish. A merely negligible presence of intact *Y. ruckeri* bacteria could be detected in spleen and head kidney of AquaVac ERM-vaccinated fish. Nonimmunized *Y. ruckeri*-infected fish showed a massive occurrence of bacteria in both organs until day 7 p.i. The detected bacteria appeared either singly or in aggregated masses and were found to be widely distributed across the parenchyma, involving sinusoids and blood vessels. In spleen, single bacteria were more prevalent in the central region than in the periphery, while in head kidney, they were prevalent in the peripheral region (Table 11). None of the fish carried bacteria before challenge. qPCR analysis of *Y. ruckeri* 16S rRNA transcripts in samples from both infected organs showed bacterial loads corresponding to those found by FISH at 4 m.p.v. (Fig. 8).

Evaluation of vaccine efficacy. The efficacies of the vaccines were previously described as the relative percentages of survival (RPS) (18), where AquaVac RELERA vaccinates showed superior protection (mortality following i.p. challenge, 42.5%) and AquaVac ERM-vaccinated fish had an intermediate mortality of 63.7% but higher survival than nonimmunized fish, which had a mortality of 73.7%. In order to express vaccine efficacy on a histopathological scale, we examined four broad parameters based on our self-developed scale and scored them semiquantitatively. For easy comprehension, the pathological scores were described as overall tissue changes in each organ/fish/group examined (Fig. 9). In spleen, following infection at day 3 p.i. (at 4 m.p.v. and 8 m.p.v.), the overall tissue changes were most severe in nonimmunized *Y. ruckeri*-infected fish and mildest in AquaVac RELERA vaccinates. The magnitude of damage and reactive changes with a negative impact were maximal in *Y. ruckeri*-infected fish and least severe in AquaVac RELERA vaccinates. Three weeks after infection (at 4 m.p.v.), the vaccinated groups showed early revival of the lost organ structure, while this restructuring took place already 2 weeks after infection in fish challenged at 8 m.p.v. The susceptibility of the organ to infection was highest in nonimmunized fish, followed by AquaVac ERM vaccinates, whereas no susceptibility was observed in AquaVac RELERA-vaccinated fish at 4 m.p.v. When challenged at 8 m.p.v., it was only the nonimmunized fish which exhibited susceptibility to infection. In head kidney, overall tissue changes were comparatively milder than the spleen devia-

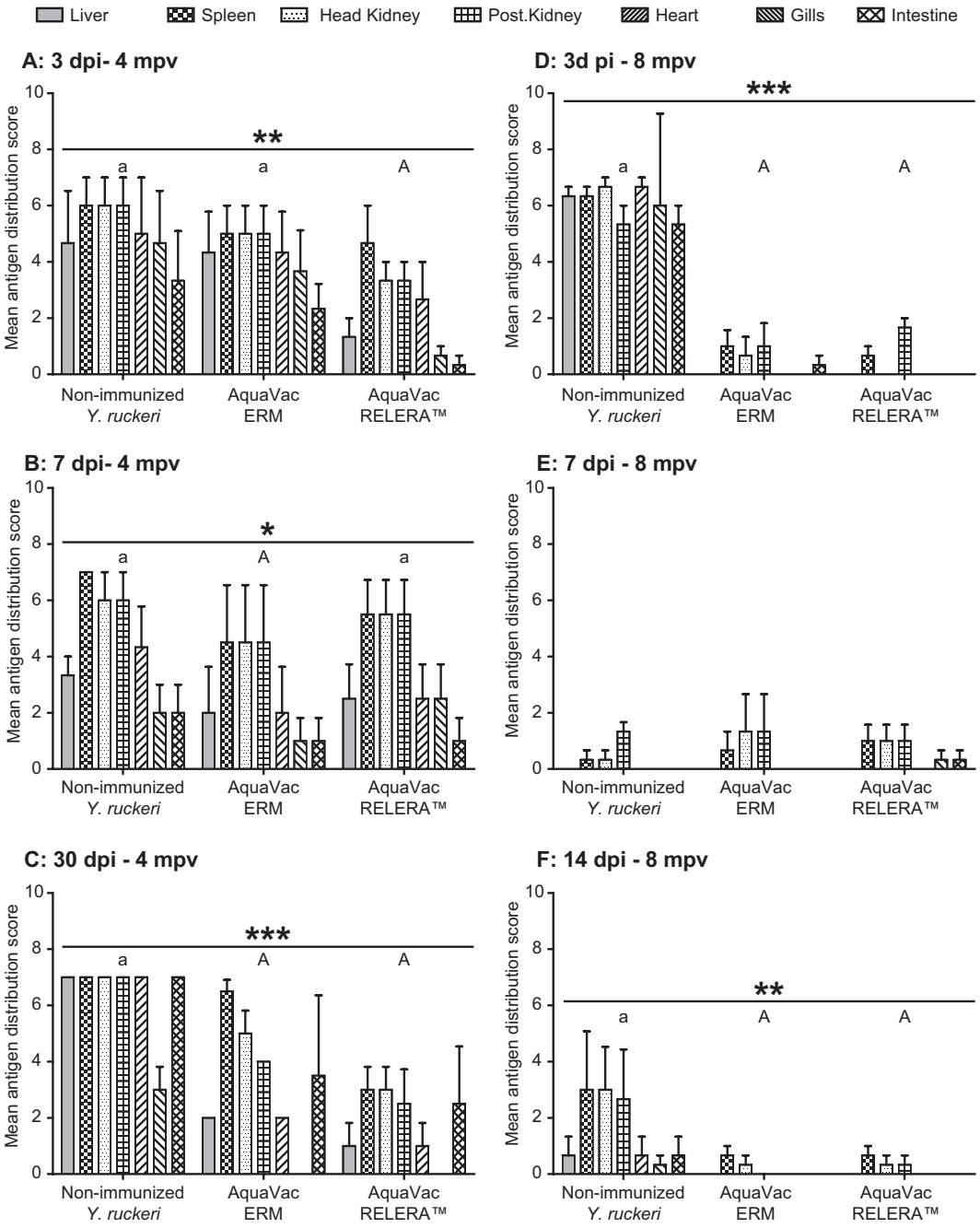

FIG 7 Mean antigen (*Y. ruckeri*) distribution in various organs from unvaccinated and vaccinated fish following infection with *Y. ruckeri* serotype O1 biotype 2 at 4 or 8 m.p.v. Results are presented as the means + SEM for 3 fish for each organ/group. Asterisks indicate significant differences among groups at the particular time point as analyzed by one-way ANOVA (*, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$). Upper- and lowercase letters indicate significant differences with respect to the sham-challenged group; same-case letters indicate no significant difference between groups as determined by Tukey's multiple comparison test.

tions. They were most pronounced in nonvaccinated fish and mildest in AquaVac RELERA-vaccinated fish. The relative levels of *Y. ruckeri* at day 3 p.i., as detected in spleen and head kidney by qPCR (*Y. ruckeri* 16S rRNA transcripts), indicated the presence of considerable amounts of bacteria in nonimmunized *Y. ruckeri*-infected fish. The levels were higher than the levels in AquaVac ERM vaccinates, which again, were higher than the bacterial levels in AquaVac RELERA vaccinates. The comparison of tissue distributions showed that spleen tissues had higher infection rates than head kidneys in all vaccinated groups (Fig. 8).

DISCUSSION

The results of the present study indicate that a number of protective immune mechanisms (including cellular and humoral elements) are involved in the fast elimination of *Y. ruckeri* in vaccinated rainbow trout following challenge. Measurements of

TABLE 11 Localization and numbers of intact *Y. ruckeri* bacteria as observed by *in situ* hybridization in spleen or head kidney from unvaccinated and vaccinated fish

Organ (time point), bacterial localization	No. of bacteria or colonies (avg ± SD [$n = 3$ fish]) in organ of fish from indicated group at indicated time point[a]							
	Nonimmunized infected		AquaVac ERM vaccinated			AquaVac RELERA vaccinated		
	3	7	0	3	7	0	3	7
Spleen (4 mpv)								
Single bacteria (peripheral region)	74.77 ± 97.5	—	—	0.44 ± 1.33	—	—	—	—
Macro-/microcolonies (peripheral region)	8.22 ± 11.1	—	—	0.11 ± 0.33	—	—	—	—
Single bacteria (central region)	93.66 ± 116	3.55 ± 10.6	—	—	—	—	—	—
Macro-/microcolonies (central region)	4.66 ± 5.0	—	—	—	—	—	—	—
Spleen (8 mpv)								
Single bacteria (peripheral region)	92 ± 103.2	—	—	—	—	—	—	—
Macro-/microcolonies (peripheral region)	14.55 ± 12.39	—	—	—	—	—	—	—
Single bacteria (central region)	120.2 ± 104	—	—	—	—	—	—	—
Macro-/microcolonies (central region)	20.22 ± 11.93	—	—	—	—	—	—	—
Head kidney (4 mpv)	3	7	0	3	7	0	3	7
Single bacteria (peripheral region)	30 ± 34.0	—	—	—	—	—	—	—
Macro-/microcolonies (peripheral region)	2.44 ± 4.4	—	—	—	—	—	—	—
Single bacteria (central region)	23.22 ± 35.3	3.55 ± 10.6	—	0.88 ± 2.66	—	—	—	—
Macro-/microcolonies (central region)	—	—	—	—	—	—	—	—
Head kidney (8 mpv)/days postinfection	3	7	0	3	7	0	3	7
Single bacteria (peripheral region)	32.88 ± 27.0	—	—	—	—	—	—	—
Macro-/microcolonies (peripheral region)	6.77 ± 5.99	—	—	—	—	—	—	—
Single bacteria (central region)	22.44 ± 22.2	—	—	—	—	—	—	—
Macro-/microcolonies (central region)	1.0 ± 1.5	—	—	—	—	—	—	—

[a] Counting fields were 0.014 mm^2. No evidence of bacteria was detected after day 7 p.i. in any group. —, none detected.

effector molecules (lysozyme and immunoglobulin) in serum and gene expression by qPCR, cellular dynamics as shown by histopathology and immunohistochemistry, and finally, pathogen elimination determined by *in situ* hybridization techniques lead us to suggest an orchestrated immune response involving cells in the central immune organs. A clear immunological memory in vaccinated rainbow trout was also documented through superior survival (18) and pathogen elimination (qPCR, FISH, and IHC) following exposure to *Y. ruckeri*. This supported previous studies demonstrating a relatively high level of protection against ERM conferred by immersion vaccination (4, 33, 34). Our comparative approach using two ERM vaccines with different efficacies and a nonimmunized infected control group allowed us to correlate protection to cellular and humoral events in the hosts. Although it would be expected that adaptive elements should play a prominent role in protection, we observed that factors often related to the innate immune response (SAA, complement factors, lysozyme, and macrophage activity) (6, 33–35) were found in vaccinated fish at higher levels than were seen in nonimmunized fish. This indicated that innate effector molecules are also of importance for pathogen elimination even in vaccinates in the early phase of infection. The involvement of a Th2-like adaptive response at later stages of infection, through the activation of genes encoding IL-4/13a, GATA-3, IgM, and IgT, was suggested in this study. This was further supported by increased occurrence of interstitial IgM and serum IgM and the proliferation of lymphocytes (B lymphocytes) in central immune organs.

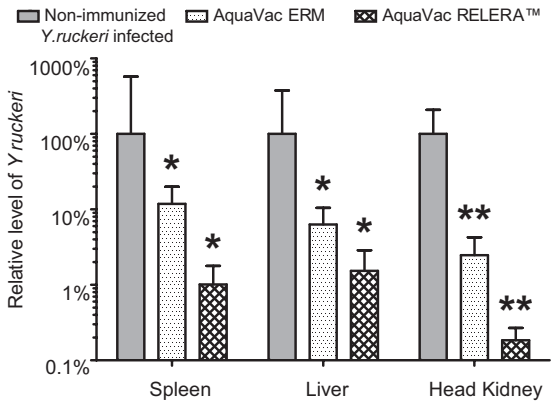

FIG 8 Relative levels of *Y. ruckeri* at day 3 p.i. in fish infected at 4 m.p.v. The expression of the ribosomal 16S gene of *Y. ruckeri* was used to detect the presence of *Y. ruckeri* in liver, spleen, and head kidney. The vaccinated groups were compared to the nonimmunized group. Note the logarithmic scale of the *y* axis (level of *Y. ruckeri*). *, $P < 0.05$; **, $P < 0.01$.

Innate immune molecules, such as serum lysozyme, showed superior activities in the highly protected group, i.e., AquaVac RELERA vaccinates, from day 3 p.i. until day 7 p.i. at 4 m.p.v. and, especially, on day 3 p.i. at 8 m.p.v. This factor is likely to restrict the propagation of infection and contribute to the observed protection with the lowest mortality in vaccinated fish (2, 6). The gene encoding lysozyme was upregulated, together with genes for complement factors (such as C3 and C5), by day 3 p.i., which suggests that at least the alternative pathway may contribute to early-phase

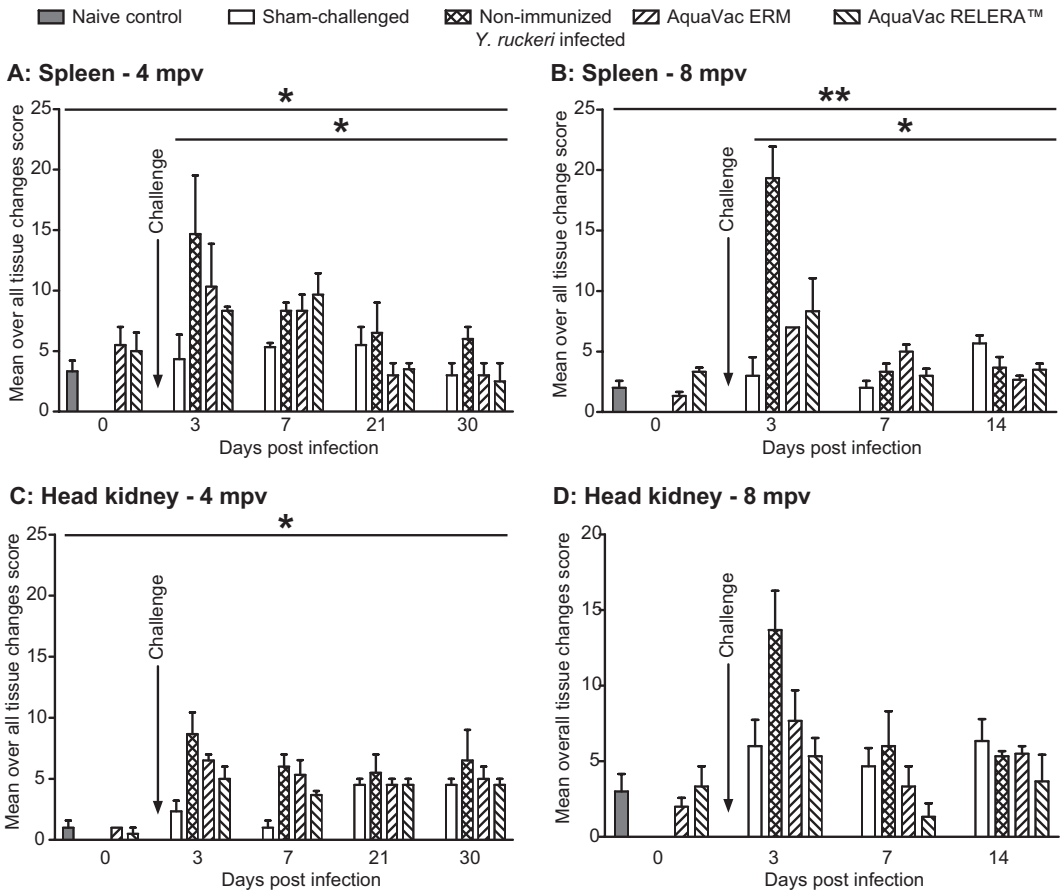

FIG 9 Overall tissue changes noted in spleen and head kidney from nonimmunized vaccinated fish following infection at 4 or 8 m.p.v. Results are presented as the means + SEM for 2 or 3 fish from each group. Asterisks indicate significant differences among groups as analyzed by Kruskal-Wallis one-way ANOVA (*, $P = 0.05$; **, $P = 0.01$).

protection. In order to determine the involvement of various immune molecules in the early protection process, we found a close association of functional regulation with complement factors and acute-phase proteins and noted a selective regulation of SAA (36). The antimicrobial peptide CATH 2, whose expression usually marks the progression of infection (37), was found to be at lower levels in AquaVac RELERA-vaccinated fish than in nonimmunized infected fish during the early infection phase, which may reflect the lower infection levels in vaccinates.

The expression levels of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α, were found to be markedly increased in nonimmunized *Y. ruckeri*-infected fish immediately following infection (at day 3 p.i.) compared to their expression levels in vaccinated fish (3). This suggests that the higher bacterial load in these hosts is responsible for inducing this immediate response. The associated early production of the potent antiinflammatory IL-10 proposes a regulatory action of IL-10 in alleviating/counteracting short-acting damage inflicted due to inflammation.

Considering the adaptive arm of immunity, we found an inclination for T helper cell (CD4)-mediated activity instead of T cytotoxic cell ($CD8^+$) activity in the early phase of infection. The low CD8 gene expression noticed in the early stage of infection (i.e., at day 3 p.i. and day 7 p.i.) was markedly correlated with our IHC results, where we noticed the disappearance of CD8α cells from spleen and head kidney in heavily infected (nonimmunized *Y. ruckeri*-infected) and moderately protected fish (AquaVac ERM-vaccinated fish). A similar observation has also been made during mammalian *Yersinia* infections, where the *Yersinia* outer protein P (YopP) was found to inhibit the CD8 T cell response in mice (38). AquaVac RELERA vaccinates did not exhibit such a disappearance of cells by IHC, and the initial low regulation of the CD8 gene was quickly restored to positive regulation by day 7 p.i. We noticed positive regulation of GATA-3 gene expression and modest downregulation of the T-bet gene in infected groups (especially in AquaVac RELERA vaccinates on day 7 p.i.) at many time points, suggesting a Th2-like polarization of T cell activity in the given cases. Realizing that *Y. ruckeri* infection downregulates these two transcription factors for evasion of host immune responses (39), we considered whether the observed regulation in AquaVac RELERA vaccinates could be a potential correlate of protection following vaccination.

It is believed that in murine yersiniosis, protective-cell-associated defense mechanisms comprise macrophages/NK cells, which become activated via T helper cells (CD4) producing significant amounts of IFN-γ (40, 41). In rainbow trout lymphoid organs, we found high expression of both IFN-γ and TNF-α genes in nonimmunized *Y. ruckeri*-infected fish and moderate regulation in vaccinated fish, especially on day 3 p.i. Together with the histological

evidence of macrophage-like transformation of hypertrophied sinusoidal and stromal cells (in spleen) and medium-sized reniform-to-cleaved nucleated lymphocytes (in head kidney), we suggest that similar events may occur in trout to restrict and eliminate the pathogen. Bacterial infections in higher vertebrates elicit alternative macrophage activation, which contributes to tissue remodeling that restricts pathogen propagation (42). Similar events may occur in trout, especially around ellipsoids/arterioles in spleen and as stellate/spindle-shaped fibrous reticular cells in head kidney. The increased expression of IL-4/-13a and IgM genes in liver tissue of vaccinated fish further adds to the notion that a Th2-like response may take place and contribute to protection against infection more quickly and strongly in vaccinated fish than in nonimmunized *Y. ruckeri*-infected fish. The low and differing levels of circulating antibody in various infected groups in the beginning of infection indicate that the protection noted in the early phase of infection is not only due to an adaptive humoral response but may include innate immunity elements, such as lysozyme and SAA, until the antibody production has been upgraded. Thus, antibodies were present at lower levels during the initial challenge, but 14 days later, the levels were maximized. This was also illustrated by the results of histology and IgM immunostaining, which showed repopulation of lymphoid cells along with the secreted form of IgM in spleen and head kidney. The $CD8\alpha^+$ cell density also increased gradually and was high in all infected groups at day 30 p.i. (at 4 m.p.v.) and day 14 p.i. (at 8 m.p.v.). Maximal presence of the IgM secretory form cooccurred with maximum $CD8\alpha^+$ cell densities in AquaVac RELERA-vaccinated fish at both 4 and 8 m.p.v. This suggests that T cell cytotoxicity may also act as a protective factor in trout during the later phase of infection.

After challenge, the degrees of tissue changes noted in vaccinated fish, which were most pronounced in the first week, were relatively lower than in the nonimmunized groups. When the two vaccines were compared, the fish vaccinated with AquaVac RELERA showed less-pronounced changes with faster restructuring in both spleen and head kidney, which reflects the better protection in this group (18).

An additional important observation in the study was the concurrent disappearance of melanomacrophage cells with invading *Y. ruckeri* infections on day 3 p.i. in spleen. This could indicate a possible utilization of melanomacrophage aggregates during pathogen elimination. The subsequent repopulation on day 7 p.i. suggests a role of these cells during primary responses to infection. This phenomenon was also found in the vaccinated group but with a moderate degree of disappearance, which may be interpreted as a priming of vaccinated fish, leaving them more effective in pathogen clearance.

It was further indicated that age-related innate factors supplemented fish resistance to infection at 8 m.p.v., as reflected by higher natural resistance to infection, lower levels of pathological alterations, quick waning of lesions, higher levels of serum lysozyme activity, and antibody responses after infection in all groups.

The histopathological approach that we adopted has indicated a dynamic and complex interplay of different cells in central immune organs during infection and response. The cellular events were correlated with the expression of immune genes and elevation of humoral factors in the host during pathogen elimination.

Conclusion. We visualized the cellular reactions connected with immune protection in both vaccinated and nonimmunized rainbow trout. The resident cells mainly of spleen but also of head kidney showed marked plasticity and ability to transform into the various cell types discussed (macrophage/epithelioid/lymphostromal). This suggests that they are engaged in elimination of the pathogens invading the fish. Such a process could be associated with the observed elevated armament of T cells and B cells. It may be hypothesized that, together with a series of antimicrobial humoral molecules, including specific antibodies and, to some extent, SAA, complement factors, and lysozyme, these events may eventually lead to pathogen elimination. Based on these observations of correlations, it will be relevant to perform a series of assays in order to describe the causality between the occurrence of individual immune cells (alone and in combination) and pathogen clearance.

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